IMPLICATIONS D'UN COMPLEXE NAD(P)H DESHYDROGENASE ET D'UNE OXYDASE TERMINALE CHLOROPLASTIQUES DANS LA PHOTOSYNTHESE ET LA CHLORORESPIRATION

Thierry Joet

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Thierry JOËT

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JURY

Mme. F. Casse, Professeur, Université Montpellier II	Examinateur
M. Z. Cerovic, Chargé de recherche, CNRS, Orsay	Rapporteur
M. P.J. Nixon, Professeur, Imperial College of Science, London	Rapporteur
M. G. Peltier, Ingénieur, CEA Cadarache	Examinateur
M. F.A. Wollman, Directeur de recherche, CNRS, IBPC Paris	Examinateur

Résumé

Chez les plantes supérieures, les membranes thylacoïdiennes des chloroplastes contiennent des protéines de type respiratoire, un complexe NADH déshydrogénase (Ndh) homologue au complexe I bactérien et une oxydase (PTOX) homologue à l'oxydase alternative des mitochondries. Dans le but d'élucider la fonction du complexe Ndh, le gène chloroplastique *ndhB* a été inactivé par transformation chloroplastique du tabac. Chez les plantes transplastomiques obtenues, des mesures de fluorescence de la chlorophylle ont permis de démontrer que ce complexe est impliqué dans la réduction non photochimique du pool de plastoquinones (PQ). Chez les mutants dépourvus de complexe Ndh, un retard de croissance ainsi qu'une altération des capacités photosynthétiques ont été observés dans des conditions où la photorespiration est stimulée par une limitation de la disponibilité en CO_2 (par exemple un déficit hydrique modéré). En étudiant les effets de l'antimycine A, un inhibiteur du transfert cyclique des électrons autour du PS I, sur l'activité photosynthétique de ces plantes, nous avons conclu que le complexe Ndh est impliqué dans une voie de transfert cyclique et participe à la fourniture en ATP nécessaire à la fixation de CO_2 quand la demande est forte.

Afin de déterminer le rôle de PTOX, nous avons généré des plantes de tabac surexprimant cette protéine. Des mesures de fluorescence de la chlorophylle nous ont permis de démontrer l'implication de PTOX dans l'oxydation non photochimique du pool de PQ. Nous avons conclu que le complexe Ndh et PTOX, tous deux localisés dans les thylacoïdes lamellaires, constituent avec le pool de plastoquinones les éléments d'une chaîne de transfert d'électrons de type chlororespiratoire. Nous avons proposé un mécanisme par lequel l'activité du transfert cyclique des électrons autour du PS I serait régulée par l'activité de la chlororespiration, à travers un contrôle fin du niveau rédox de certains transporteurs de la chaîne de transfert d'électrons.

Mots-clés : photosynthèse, transfert cyclique des électrons autour du PS I, chlororespiration, oxydase terminale , complexe NADH déshydrogénase.

Laboratoire d'Ecophysiologie de la Photosynthèse
Département d'Ecophysiologie Végétale et de Microbiologie - Direction des Sciences du Vivant
CEA Cadarache
13108 Saint Paul lez Durance - France

Abstract

Thylakoid membranes of higher plants contain respiratory-type proteins of unknown function, namely a NADH dehydrogenase complex (Ndh) and an oxidase (PTOX), respectively homologuous to mitochondrial complex I and alternative oxidase. In order to elucidate the function of the Ndh complex, the *ndhB* gene has been inactivated by plastid transformation. Chlorophyll fluorescence measurements, performed on transplastomic tobacco plants, showed that the Ndh complex is involved in the non-photochemical reduction of the PQ pool. In mutants deficient in the Ndh complex, both a growth retardation and a decrease in photosynthetic capacities has been measured in conditions where photorespiration is highly active, due to a decreased CO_2 avaibility (such as limited water deficit). Based on the effects of antimycin A, an inhibitor of cyclic electron transport, on photosynthesis and on measurments of PS I energy storage by photoacoustic spectroscopy, we conclude to the existence of two pathways of cyclic electron flow around PS I, one of which involving the Ndh complex. This mechanism, regulated by the redox state of electron transport chain putatively through the plastoquinol oxidase activity of PTOX, could re-equilibrate the NADPH/ATP ratio when ATP demand is high, for instance during water deficit.

In order to determine the role of PTOX during photosynthesis, tobacco plants overexpressing this protein have been generated. Based on chlorophyll fluorescence measurements, we show the involvement of PTOX in the non-photochemical oxidation of plastoquinones. We conclude that the Ndh complex and PTOX, both located in stroma lamellae, are components of a chlororespiratory electron transport chain. We propose that chlororespiration, through a fine tuning of the redox state of some inter-system electron carriers, participates to the regulation of cyclic electron transport around PS I.

Key-words : photosynthesis, cyclic electron flow around PS I, chlororespiration, plastid terminaloxidase, plastid Ndh complex.

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Liste des abréviations

ABA :	acide abscissique
ADN :	acide désoxyribonucléique
ADNc :	ADN complémentaire
ADP	adénosine diphosphate
ARN :	acide rinonucléique
ARN m :	ARN messager
ATP :	adénosine triphosphate
BSA:	albumine sérique bovine
Cab :	chlorophyll a/b binding protein
chl :	chlorophylle
CO_2 :	dioxyde de carbone
Cyt *b_6/f*	Complexe protéique contenant le Cytochrome b_6 et le cytochrome *f*
kDa :	kilodalton
DBMIB:	2,5-dibromo-3-méthyl-6-isopropyl-*p*-benzoquinone
DCMU:	3-(3,4-dichlorophényl)-1,1-diméthyl urée
DTT :	dithiothréitol
EDTA :	ethylene diamine tetra acetic acid
FAD:	flavine adénine dinucléotide
FeS:	Fer/soufre
Fm:	fluorescence maximale de la chlorophylle
F_0:	fluorescence minimale de la chlorophylle
Fd:	ferrédoxine
FMN:	flavine mononucléotide
FNR:	ferrédoxine-$NADP^+$ oxydoréductase (EC 1.18.1.2)
FQR:	ferrédoxine-plastoquinone réductase
Fs	fluorescence de la chlorophylle à l'état stationnaire
Fv	fluorescence de la chlorophylle
Hepes :	acide N-[2-hydroxyéthyl] piperazine-N'[2-éthane] sulfonique
HQNO:	2-heptyl-4-hydroxyquinoline *N*-oxyde
LHC:	complexe collecteur d'énergie lumineuse
LHC I ou II:	complexe chlorophylle/proteine collecteur d'énergie lumineuse du photosystème I ou II
NAD(P) :	nicotinamide adénine dinucléotide (phosphate)
NAD(P)H :	nicotinamide adénine dinucléotide (phosphate) réduit
Ndh:	complexe NAD(P)H déshydrogénase chloroplastique

OAA	acide oxaloacétique
P_{680}:	centre réactionnel du PS II
P_{700}:	centre réactionnel du PS I
PAGE:	gel électrophorétique de polyacrylamide
PEG :	polyéthylène glycol
pb:	paires de bases
PC:	plastocyanine
PCR:	amplification en chaîne par polymérase
3-PGA	acide 3-phosphoglycérique
PMSF:	fluoride de phényl méthyl sulfate
PQ:	plastoquinone
PQH_2:	plastoquinol (Plastoquinone réduite)
PSI et PSII:	photosystème I et Photosystème II
PTOX:	oxydase terminale chloroplastique
Rubisco:	ribulose-1,5-bisphosphate carboxylase oxygénase
RuBP :	ribulose 1,5 bisphosphate
SDS:	Dodécyl sulfate de sodium
SHAM:	Acide salicylhydroxamique
SOD :	superoxyde dismutase
Tris :	Tris [hydroxyméthyl] aminométhane ou trizma base

Sommaire

Introduction

Chez les végétaux supérieurs, la photosynthèse oxygénique et la respiration sont des mécanismes bioénergétiques d'oxydo-réduction qui mettent en jeu des chaînes de transfert d'électrons couplées à la synthèse d'ATP. La respiration est le mécanisme par lequel les sucres et les lipides sont oxydés en CO_2 et H_2O et l'énergie contenue dans les liaisons moléculaires est convertie en ATP. La fonction photosynthétique, quant-à-elle, utilise l'énergie lumineuse pour réaliser la photolyse de l'eau et permettre la production de pouvoir réducteur (NADPH) et d'ATP, nécessaires à la synthèse de molécules organiques à partir du CO_2. Photosynthèse et respiration possèdent des bilans de matière antagonistes et sont confinées dans des organites spécialisés, respectivement le chloroplaste et la mitochondrie, semi-autonomes du point de vue génétique. Les chaînes de transfert d'électrons chloroplastiques et mitochondriales présentent une organisation assez similaire comprenant des composants homologues tels que les quinones, les ATP synthases ou certains cytochromes. Selon la théorie endosymbiotique, les chloroplastes dériveraient de l'endosymbiose entre une cellule eucaryote et une cyanobactérie primitive (pour une synthèse, voir Gray, 1991). Chez les procaryotes photosynthétiques et les cyanobactéries, les réactions d'oxydo-réduction photosynthétique et respiratoire ont lieu au sein de la même membrane et certains des complexes protéiques sont communs aux deux chaînes de transporteurs d'électrons (Verméglio et Joliot, 1984 ; Scherer, 1990).

Au cours de la photosynthèse oxygénique, les deux photosystèmes PS II et PS I, présents dans la membrane thylacoïdienne, fonctionnent d'une manière couplée et permettent un transfert linéaire d'électrons de l'eau vers le $NADP^+$, accepteur terminal d'électrons. En plus des réactions impliquées dans ce transfert, appelé schéma en "Z", des voies dites « alternatives » ont été décrites chez les algues unicellulaires et dans les chloroplastes des plantes supérieures. Parmi celles-ci, il a été suggéré qu'un transfert cyclique des électrons autour du photosystème I permette la synthèse d'ATP par le jeu des photophosphorylations cycliques (pour une synthèse, voir Bendall et Manasse, 1995). D'autre part, l'existence d'une chaîne de type respiratoire, dénommée chlororespiration, impliquant le pool de plastoquinones (PQ) et une oxydase terminale, a été suggerée au sein des chloroplastes

(Bennoun, 1982; Peltier et al., 1987). Ces voies alternatives de transfert d'électrons impliquent une étape de réduction non-photochimique du pool de PQ indépendante du PS II au cours de laquelle le NAD(P)H stromal pourrait servir de donneur d'électrons et une voie d'oxydation non-photochimique des plastoquinones. Ces voies alternatives sont encore très mal connues tant du point de vue de la nature moléculaire des transporteurs d'électrons impliqués que de leur rôle physiologique. L'objectif de ce travail de thèse visait à répondre à ces deux questions.

Analyse bibliographique

I. Le chloroplaste et la photosynthèse

A. Organisation générale des chloroplastes

Les organismes photosynthétiques se caractérisent par leur capacité à utiliser l'énergie lumineuse pour synthétiser de la matière organique, au cours d'un processus biochimique appelé photosynthèse. Chez les végétaux, la photosynthèse se déroule au sein d'un organite spécialisé, le chloroplaste (Figure 1).

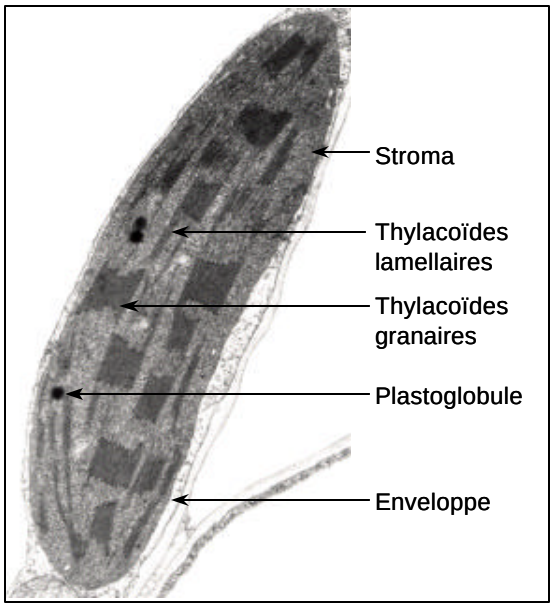

Figure 1. Micrographie par transmission électronique d'un chloroplaste de *Nicotiana tabacum* (5000x). Cliché aimablement fourni par F. Eymery (CEA Cadarache).

Cet organite est délimité par une double enveloppe, composée d'une membrane externe, perméable aux métabolites de faible poids moléculaire, et d'une membrane interne délimitant une matrice intra-chloroplastique, le stroma. Il est communément admis que le chloroplaste des plantes supérieures dérive d'un hôte procaryotique endosymbiotique, ancêtre de la cyanobactérie (pour une synthèse, voir Whatley, 1993). La capture de l'énergie lumineuse et sa conversion en énergie chimique s'opère au sein des membranes thylacoïdiennes internes au chloroplaste et formant un réseau composé d'empilements de petites vésicules aplaties, les thylacoïdes granaires, interconnectés par des thylacoïdes lamellaires. Ces membranes contiennent les pigments photosynthétiques responsables de la capture de l'énergie lumineuse, et les protéines nécessaires à sa conversion en énergie chimique. La photosynthèse permet la conversion du

CO_2 atmosphérique en carbone organique grâce à l'énergie lumineuse. Le bilan de la photosynthèse est : $6\,CO_2 + 6\,H_2O \longrightarrow C_6H_{12}O_6 + 6\,O_2$.

La photosynthèse est un phénomène complexe qui peut être décrit en deux phases (Figure 2). La première phase, appelée phase photochimique, se déroule au sein des membranes thylacoïdiennes et permet la capture de l'énergie lumineuse et sa conversion en énergie chimique, sous forme de liaison phosphoanhydre contenue dans l'ATP et de pouvoir réducteur par la réduction du $NADP^+$ en NADPH. La seconde phase, appelée phase non photochimique, se déroule dans le stroma. Elle correspond à la fixation du CO_2 et à sa transformation en carbone organique grâce à l'utilisation de l'énergie chimique contenue dans l'ATP et le NADPH, au cours du cycle de Calvin.

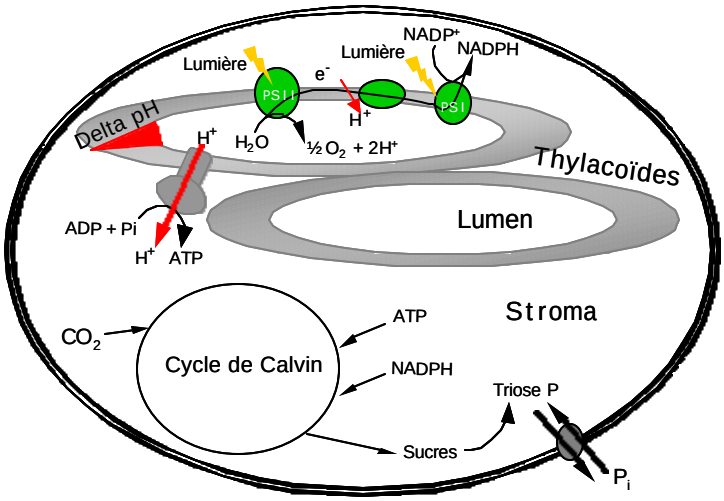

Figure 2. Représentation schématique des processus photosynthétiques au sein du chloroplaste. La phase photochimique se déroule au sein des membranes des thylacoïdes, la phase non photochimique (cycle de Calvin) dans le stroma. PS : photosystème.

B. Absorption de l'énergie lumineuse et génération de pouvoir réducteur

L'énergie lumineuse est absorbée par les chlorophylles et les caroténoïdes organisés en antennes collectrices ou LHC (Light Harvesting Complex), puis transférée sous forme d'énergie d'excitation (exciton) vers les centres réactionnels des photosystèmes I et II. Ceux-ci réalisent alors une séparation de charges par délocalisation d'un électron au niveau d'une paire de molécules de chlorophylle (chl *a**). Cet électron est transféré à la chaîne photosynthétique et l'accumulation de charges positives du côté donneur du PS II permet de réaliser la photolyse de l'eau, entraînant une production d'oxygène (Figure 3). Du côté accepteur du PS II, les électrons sont transférés dans une chaîne de transporteurs membranaires constituée des plastoquinones, du complexe cytochrome b_6/f, de la plastocyanine (protéine soluble du lumen dont la forme réduite constitue le donneur au PS I).

Le PS I réalise également une séparation de charge entraînant l'oxydation des plastocyanines. Du coté donneur du PS I, les électrons sont transférés à la ferrédoxine, protéine soluble du stroma. Enfin, la ferrédoxine-NADP réductase réalise la réduction du $NADP^+$ à partir de la ferrédoxine réduite au niveau du PS I.

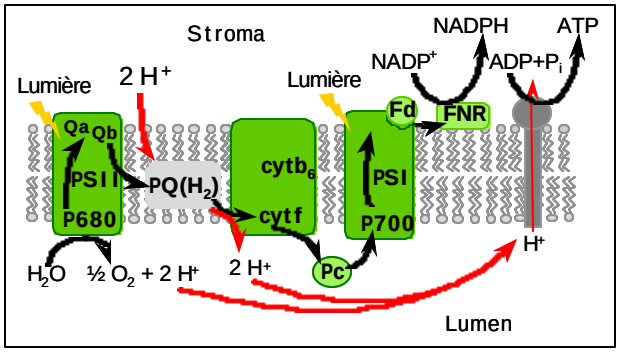

Figure 3. Schéma du transfert photosynthétique d'électrons (shéma en Z) et des translocations de protons. Les transferts d'électrons sont représentés en noir, les translocations de protons en rouge. PS : photosystème, $PQ(H_2)$: Plastoquinones ou (plastoquinols), Pc : plastocyanine, Fd : ferrédoxine, FNR : ferrédoxine $NADP^+$ réductase. Cyt : cytochrome.

Le transfert d'électrons entre les transporteurs de la chaîne photosynthétique s'effectue dans le sens du potentiel rédox, hormis les deux sauts énergétiques réalisés aux niveaux des photosystèmes I et II, permis par l'énergie lumineuse (pour une synthèse voir Blankenship et Prince, 1985). L'ensemble des réactions qui réalisent le transfert linéaire des électrons de H_2O vers le $NADP^+$ grâce à un couplage entre PSII et PS I constitue le « schéma en Z » de la photosynthèse (Figure 3) (Hill et Bendall, 1960).

C. Les composants de la chaîne de transfert d'électrons

Le PS II est constitué d'un centre réactionnel, appelé P_{680} contenant la chlorophylle *a* (chl *a*) et de nombreuses protéines. Parmi celles-ci, les protéines D_1 et D_2, qui consistuent le cœur du centre réactionnel, sont codées par les génes chloroplastiques *psb*A et *psb*B et présentent des structures primaires voisines (Robinson, 1996) (Figure 4). D_1 et D_2 contiennent chacune cinq hélices transmembranaires et forment un hétérodimère (Barber, 1987). Chaque protéine D_1/D_2 contient un résidu tyrosine qui sert de donneur d'électrons à la chlorophylle a du P_{680}^+ (Debus et al., 1988). Les protéines D_1 et D_2 sont associées avec une phéophytine et une plastoquinone (Q_A) (Melis, 1991). Le PS II est associé à des antennes collectrices de lumière ou LHCII (Kühlbrandt et Wang 1991 ; Hankamer et al., 1997). La plupart des pigments des LHC sont associés à des protéines dénommées protéines CAB (Chl a/b binding protein) et forment les complexes protéine-chlorophylle les plus abondants. L'énergie d'excitation est transmise des LHCII au centre réactionnel, P_{680}, par l'intermédiaire des protéines antennes CP_{47} et CP_{43} (codées par les gènes *psb*C et *psb*D, Diner et Babcock, 1996).

Le cytochrome b_{559} est est composé de deux sous-unités de 9 kDa (α) et 4,5 kDa (β) codées respectivement par les gènes *psb*E et *psb*F. Bien qu'il ait été sugggéré qu'il participe à de nombreux processus, comme l'assemblage du PS II, la photolyse de l'eau, la protection contre la photoinhibition ou le transfert cyclique des électrons autour du PSII, sa fonction précise demeure inconnue (Whitmarsh et Pakrasi, 1996).

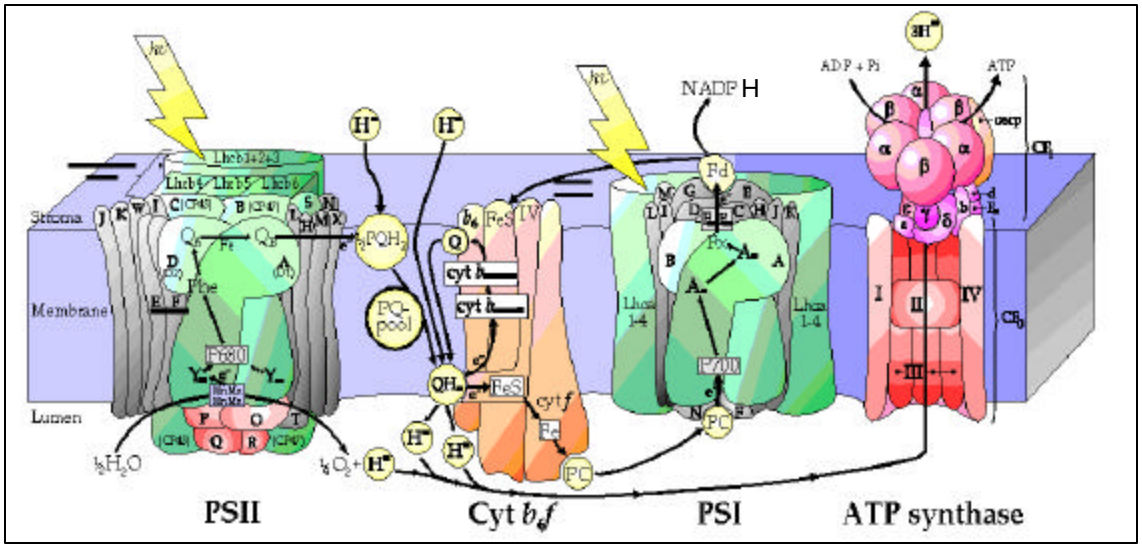

Figure 4. Représentation schématique des différentes sous-unités des complexes photosynthétiques au sein des membranes thylacoïdiennes (d'après Hankamer et al., 1997 et Nield J., Thèse, University of London, 1997).

Plusieurs autres protéines de petite taille dont le rôle est encore inconnu, codées par les gènes *psb* H, J, K, L, M, R, participent à la structure du PS II (Diner et Babcock, 1996). Enfin, la plastoquinone Q_B, également associée au PSII, transfère les électrons de Q_A vers le pool de plastoquinones liposolubles.Enfin, trois protéines extrinsèques de 33, 24 et 17 kDa (codées respectivement par les gènes *psb*O, *psb*P et *psb*Q) et des cofacteurs inorganiques tels que le manganèse, le calcium et le chlore sont impliqués dans la photolyse de l'eau (Renger, 1997).

Les plastoquinones (environ 7 molécules de PQ par PS II) permettent le transport d'électrons entre le PS II et le complexe cytochrome b_6/f. Grâce à leur queue hydrocarbonée composée d'unités isopréniques, les plastoquinones sont solubles dans le cœur hydrophobe des bicouches phospholipidiques et y sont mobiles. En acceptant un électron, la PQ est réduite en semi-quinone PQ^{-}, forme semi-radicalaire dans laquelle l'électron célibataire est délocalisé entre le noyau benzènique et les atomes d'oxygène. En acceptant un deuxième électron et deux protons, la PQ est entièrement réduite en PQH_2 (plastoquinol). Sous cette forme, elle peut servir de donneur d'électrons au complexe cytochrome b_6/f.

Le complexe cytochrome *b6/f* présente quatre centres rédox liés par 4 composants protéiques majeurs, une protéine à centre fer-soufre, dite protéine de Rieske, le cytochrome *f*, le cytochrome b_6 et la sous-unité IV (Lemaire et al., 1986) (Figure 4). Cette dernière est impliquée dans la liaison avec les PQH_2 (Hauska et al., 1996). Le site Q_o situé à proximité de la face du lumen est le site où a lieu l'oxydation des plastoquinols PQH_2, qui s'accompagne de la réduction du centre fer-soufre de la protéine de Rieske (Anderson, 1992; Cramer et al., 1996). Quatres autres sous-unités mineures de 3 à 6 kDa dont le rôle est inconnu sont associées au cytochrome *b6/f* . Enfin, l'hème du cytochrome *f* (hème C), réduit par la protéine de Rieske permet le transfert des électrons vers la plastocyanine (Allred et Staehelin, 1985). Le cytochrome b_6, partie la plus hydrophobe du complexe (contenant deux hèmes b, b_{6H} et b_{6L}) est également réduit lors de l'oxydation des plastoquinols au site Q_o. Le cytochrome *b6/f* possède un deuxième site de liaison aux PQs, le site Q_i situé à proximité de la face stromale. Ce site est impliqué dans un transfert cyclique d'électrons au sein du cytochrome *b6/f* appelé cycle Q proton-moteur (voir paragraphe I.D).

La plastocyanine (PC) est une protéine soluble du lumen de 10 kDa codée par le génome nucléaire. Relativement acide et contenant un atome de cuivre, la PC est mobile dans le lumen. Réduite par le cytochrome *f*, elle réduit à son tour le centre réactionnel du PSI (P_{700}^+). Le photosystème I est constitué d'un centre réactionnel (appelé P_{700}) et d'antennes collectrices (LHC I) (Knaff, 1988). Le centre réactionnel contient une dizaine de sous-unités polypeptidiques, 40 à 65 molécules de chlorophylle a, une ou deux molécules de β-carotène, deux molécules de vitamine K_1 et trois centres fer-soufre (Nechushtai et al., 1996) (Figure 4). Les LHC I sont des composants protéines-chlorophylles minoritaires représentant 5 à 10% des pigments membranaires des thylacoïdes. Les deux protéines majeures du PSI, Psa A et Psa B sont codées par le génome chloroplastique. Des protéines à centres fer-soufre F_x et F_A/F_B sont liées au centre réactionnel et forment les premiers accepteurs stables du PS I. Les sous-unités Psa C, D, E, H sont impliquées dans l'organisation, la stabilisation du PS I et participent au transfert d'électrons vers la ferrédoxine, premier transporteur d'électrons soluble dans le stroma. La ferrédoxine est une protéine de 11 kDa codée par le génome nucléaire, constituée d'un centre fer-soufre binucléaire.

Enfin, la ferrédoxine $NADP^+$ réductase (FNR), protéine hydrophyle de 35 kDa codée par le génome nucléaire, assure la réduction du $NADP^+$ à partir de la ferrédoxine réduite. La FNR appartient à une classe importante de flavoenzymes qui utilisent une flavine adénine dinucléotide (FAD) liée de façon non covalente comme seul centre d'oxydoréduction (Karplus et al., 1991).

D. Gradient de protons trans-membranaire et synthèse d'ATP

Un gradient transmembranaire de protons est généré par le transfert photosynthétique d'électrons. En premier lieu, la photolyse de l'eau au niveau du PSII libère dans le lumen deux protons par paire d'électrons transférés à la chaîne ($H_2O \rightarrow 2\ e^- + 2H^+ + \frac{1}{2}O_2$). Au cours de leur réduction, les plastoquinones empruntent deux protons côté stroma et les libèrent côté lumen lors de leur oxydation sur le site Q_o du cytochrome b_6f (Deniau et Rappaport, 2000). Les plastoquinones servent ainsi de transporteur de protons à travers la membrane des thylacoïdes ($PQ_{ox} + 2e^- + 2H^+ \leftrightarrow 2\ PQH_2$) (Cox et Olsen, 1982). De plus, deux protons sont transportés du stroma vers le lumen par le cytochrome b_6/f au cours du cycle Q (Figure 5) (Cramer et al.,1996). Ce processus fonctionne sur le même modèle que le cycle Q mitochondrial qui a lieu au niveau du cytochrome *b/c* (Mitchell, 1975 ; Slater, 1983 ; Tang et trumpower, 1986). Au site Q_i, au niveau de la face stromale de la membrane thylacoïdienne, une plastoquinone oxydée sert d'accepteur d'électrons du cytochrome b_{6H}. Ce transfert produit une semiquinone (anion $PQ^{\cdot-}$) qui sert d'accepteur pour un second électron provenant également du cytochrome b_{6H}. La plastoquinol PQH_2 ainsi formée diffuse à travers la membrane et cède ses protons dans le lumen au site Q_o. En même temps, deux électrons sont cédés par PQH_2, l'un au cytochrome b_{6L}, l'autre à la protéine de Rieske et enfin au cytochrome *f* de l'espace intermembranaire. La plupart des études mettant en évidence l'existence du cycle Q ont été menées *in vitro* sur des thylacoïdes isolés ou des chloroplastes (Selak et Whitmarsh, 1982 ; Hope et Matthews, 1988; Osborne et Geider, 1988). Certains auteurs considèrent ce mécanisme comme obligatoire pour chaque passage d'électrons (Davenport et McCarty, 1984; Rich, 1988 ; Kobayashi et al., 1995) et d'autres comme facultatif (Ort, 1986). Certains auteurs ont proposé que le cycle Q était inhibé sous forte lumière (Fowler et Kok, 1976 ; Graan et Hort, 1983 ; Berry et Rumberg, 1999) et d'autres que le cycle Q était actif sous

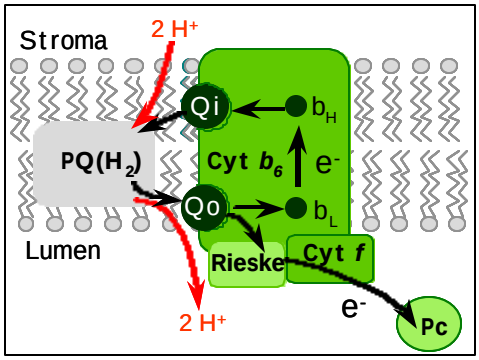

Figure 5. Schéma du transfert cyclique des électrons ou cycle Q proton-moteur au sein du cytochrome b_6/f (d'après Cramer et al., 1996). Les transferts d'électrons sont représentés en noir, les translocations de protons en rouge. Qo : site d'oxydation des plastoquinols, Qi : site de réduction des plastoquinones, b_L : hème à bas potentiel du cytochrome *b*, b_H : hème à haut potentiel du cytochrome *b*, Cyt *f* : cytochrome *f*.

certaines conditions (Furbacher et al., 1989 ; Berry et Rumberg, 1999). Récemment, l'analyse *in vivo* des cinétiques de relaxation du shift électrochromique des caroténoides en réponse aux variations du gradient électrochimique trans-thylacoïdien (Junge, 1977) a conclu au caractère obligatoire du cycle Q (Sacksteder et al., 2000).

L'énergie contenue dans le gradient de protons transmembranaire, appelée force proton-motrice, est utilisée pour la synthèse d'ATP, grâce à un complexe protéique, l'ATP synthase, qui utilise l'ADP et le phosphate inorganique (Pi) (Hammes, 1983). Ce complexe protéique est présent chez tous les êtres vivants, dans la membrane plasmique des procaryotes et dans les membranes mitochondriales et thylacoïdiennes des eucaryotes photosynthétiques. L'ATP synthase utilise le flux de protons pour générer une liaison phosphoanhydride à haute énergie entre ADP et P_i. L'ATP synthase est un complexe multi-enzymatique qui contient deux parties : les facteurs de couplage CF_1 et CF_0 (Futai et al., 1989 ; Mc Carty, 1996) (Figure 4). CF_1 est une protéine hydrophile constituée de 9 sous-unités ($\alpha_3\beta_3\gamma\delta\varepsilon$) (Boekema et Lücken, 1996). Extrinsèque à la membrane, elle réalise le couplage entre synthèse d'ATP et flux de protons. CF_0, qui constitue la partie intrinsèque du complexe, est un multimère comprenant 4 motifs polypeptidiques hydrophobes présents en multiples copies. Ce complexe constitue un canal à protons et assure l'ancrage de CF_1 à la membrane. Il a été initialement proposé que le fonctionnement des ATP synthases nécessite le passage de trois protons dans le canal de l'ATP synthase pour aboutir à la synthèse d'une molécule d'ATP (Portis et McCarty, 1976 ; Hangarter et Good, 1982). Des valeurs de 4 protons par molécule d'ATP ont également été proposées par certains auteurs (Rumberg et al., 1990 ; Kobayashi et al., 1995 ; Van Walraven et al., 1996) et semblent faire l'objet d'un consensus (pour une revue, voir Haraux et Kouchkovsky, 1998).

Ainsi, au cours du transfert photosynthétique d'électrons selon le schéma en « Z », une force proton-motrice est constituée. Dans l'hypothèse où le cycle Q serait absent, le transfert linéaire de deux électrons de H_2O à $NADP^+$ conduirait à la libération de 4 H^+ dans le lumen des thylacoïdes. Dans ces conditions, le rapport entre ATP et NADPH produits lors du transfert linéraire d'électrons serait de 1. Dans l'hypothèse où le cycle Q serait un mécanisme obligatoire, ce rapport atteindrait une valeur de 1,5.

E. Organisation des membranes thylacoïdiennes

La membrane thylacoïdienne des plantes supérieures est divisée en deux domaines caractérisés par des compositions biochimiques différentes : les lamelles du stroma (thylacoïdes non empilés) et les grana (thylacoïdes empilés) (Staehelin et al., 1976; Albertsson et al., 1991) (Figure 1). Dans les zones granaires, les liaisons intermembranaires impliquent des cations divalents tels que le Mg^{2+} et sont facilitées par l'association des LHCII (Allen et Forsberg, 2001). La matrice lipidique de ces deux domaines présente des différences de fluidité et de composition protéique (Ford et al., 1982; Anderson et Anderson, 1980). Ainsi, la plus grande partie des PSII est présente dans les empilements granaires, tandis que la majorité des PSI et des ATP synthases se situent dans les lamelles (Figure 6) (Allred et Staehelin, 1985; Anderson, 1989). Le complexe b_6/f est distribué de manière plus uniforme entre les deux types membranaires. La liaison entre les deux photosystèmes est permise par la diffusion latérale des plastocyanines soluble dans le lumen et, dans une moindre mesure, par la diffusion du pool de plastoquinols. Une partie des LHCII du PSII sont mobiles, se détachent du PSII et migrent latéralement dans la membrane vers le PSI. Cette migration, appelée « changement d'état » permet une redistribution de l'énergie d'excitation entre les deux photosystèmes (Fork et Satoh, 1986; Simpson et Knoetzel, 1996). La mobilité et la diffusion des LHCII à travers les membranes dépend de leur état de phosphorylation (Fork et Satoh, 1986; Allen, 1992) et résulte de l'activité d'une kinase. L'activité de cette kinase dépend du niveau d'oxydo-réduction de la chaîne de transfert

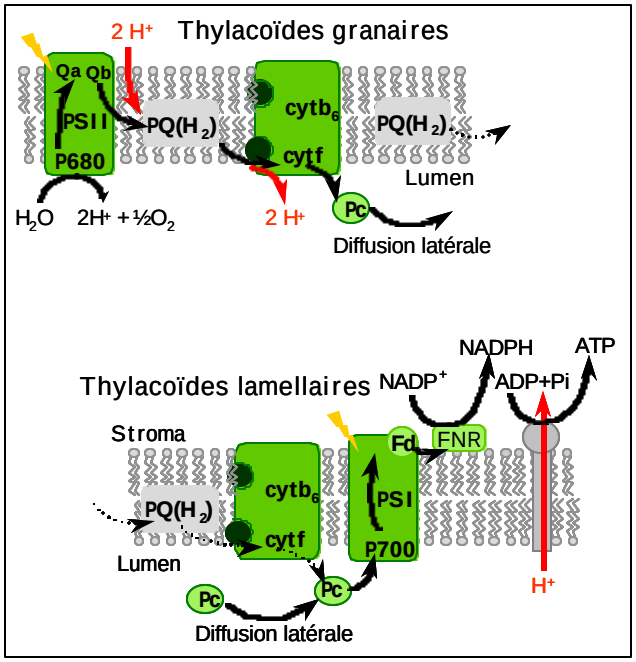

Figure 6. Schéma représentant l'organisation fonctionnelle des complexes protéiques photosynthétiques au sein des thylacoïdes granaires et lamellaires (d'après Allen et Forsberg, 2001). la liaison entre PS II et PS I est assurée majoritairement par la diffusion des plastocyanines réduites au sein du lumen. PS : photosystème, $PQ(H_2)$: Plastoquinones ou (plastoquinols), Pc : plastocyanine, Fd : ferrédoxine, FNR : ferrédoxine $NADP^+$ réductase.

d'électrons entre les deux photosystèmes et particulièrement le pool de PQ et le cyt b_6/f. Il a été rapporté que le complexe cytochrome b_6/f soit préferentiellement localisé dans les thylacoïdes lamellaires lors des transitions d'état (Vallon et al., 1991). La séparation physique des photosystèmes et la mobilité des antennes collectrices permettent de contrôler les flux d'électrons en redistribuant l'énergie entre les deux photosystèmes (Anderson, 1992; Trissl et Wilhelm, 1993).

F. Assimilation photosynthétique du CO_2

Le cycle photosynthétique de réduction du carbone ou cycle de Calvin a lieu dans le stroma et permet la synthèse de carbohydrates à partir du CO_2 et de l'énergie chimique (ATP et NADPH) produite lors du transfert photosynthétique d'électrons (Figure 7). L'enzyme clé de ce cycle, la Rubisco (Ribulose 1,5 bisphosphate carboxylase oxygénase), lie une molécule de CO_2 au ribulose 1,5 bisphosphate (RuBP) pour former deux molécules d'acide 3 phosphoglycérique (3-PGA). Cette enzyme est la protéine la plus abondante chez les végétaux supérieurs (environ 40% des protéines solubles de la feuille). Elle contient deux types de protomères, une grosse sous-unité catalytique codée par le génome chloroplastique et une petite sous-unité régulatrice codée par le genome nucléaire. Le 3-PGA formé est transformé en glycéraldéhyde 3-phosphate lors de deux réactions de phosphorylation et de réduction nécessitant de l'ATP et du NADPH. Les réactions suivantes permettent la régénération du RuBP, substrat de la Rubisco. Les trioses phosphates formés sont utilisés pour la synthèse chloroplastique d'amidon ou exportés vers le cytosol. Au cours de ce dernier processus, le glycéraldéhyde 3-phosphate est isomérisé en dihydroxyacetone phosphate, transporté du chloroplaste vers le cytosol en échange d'un ion phosphate, et sert de précurseur à la synthèse du saccharose.

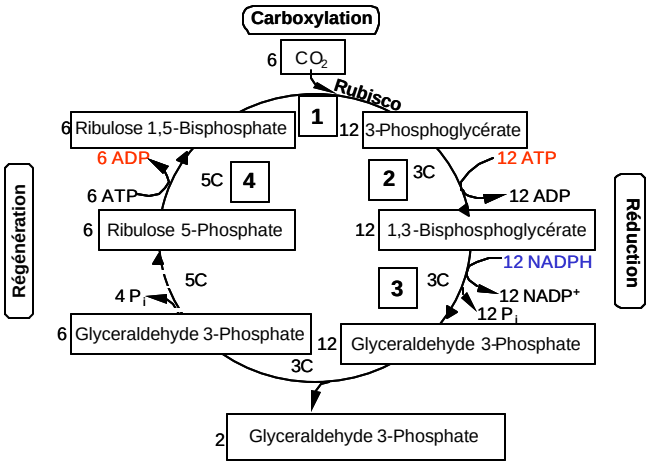

Figure 7. Cycle de Calvin ou cycle photosynthétique de réduction du carbone chez les plantes de type C_3. 1: Rubisco (RuBP carboxylase oxygénase) 2 : 3-phosphoglycérate kinase, 3 : glycéraldéhyde-3-phosphate déshydrogénase, 4 : phosphoribulokinase.

Au cours du cycle de Calvin, 18 molécules d'ATP sont hydrolysées et 12 molécules de NADPH sont oxydées. Le fonctionnement de ce cycle nécessite donc un rapport ATP/NADPH de 1,5 (Osmond, 1981).

G. Photorespiration

La Rubisco possède la particularité de fixer de manière compétitive le CO_2 et l'O_2 (Andrews et al., 1975). Ainsi, selon que son activité est de type carboxylase ou oxygénase, la Rubisco initie respectivement le cycle de Calvin ou le cycle photorespiratoire (Figure 8). La photorespiration tient son nom du fait que ce processus consomme de l'O_2 et aboutit à un rejet de CO_2 à la lumière. L'oxygénation du RuBP conduit à la formation d'une molécule de 3-PGA (disponible pour le cycle de Calvin) et d'une molécule de phosphoglycolate. Le phosphoglycolate est déphosphorylé en glycolate dans le chloroplaste, oxydé en glyoxylate dans les peroxysomes et donne lieu à la formation de glycine suite à une réaction de transamination. Au sein des mitochondries, deux molécules de glycine sont décarboxylées et donnent lieu à la formation d'une molécule de sérine, d'une molécule de NH_4^+ et d'une molécule de CO_2 (Tolbert, 1982). L'ammonium (NH_4^+), dont l'accumulation est toxique pour la cellule végétale, est ensuite recyclé par le cycle GS/GOGAT (Glutamine synthase/Glutamate synthase) au sein des chloroplastes. Après transport de la mitochondrie vers les peroxysomes, la sérine est convertie en hydroxypyruvate, qui est à son tour réduit en glycérate par l'hydroxypyruvate réductase. Le

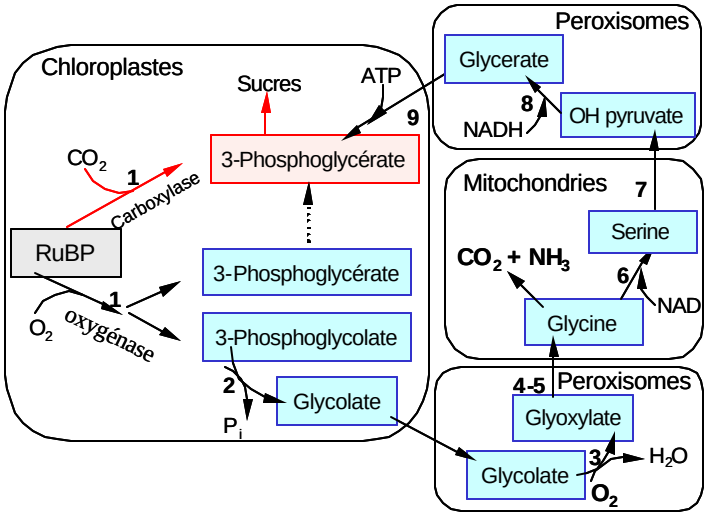

Figure 8. Cycle photorespiratoire, ou cycle photosynthétique d'oxydation du carbone (d'après Osmond, 1981). RuBP : ribulose 1,5-bisphosphate, 1: Rubisco, 2 : phosphoglycolate phosphatase, 3 : glycolate oxydase, 4 : sérine-glyoxylate aminotransférase, 5 : glutamate-glyoxylate aminotransférase, 6 : sérine hydroxyméthyl transférase, 7 : glutamate-hydroxypyruvate aminotransférase, 8 : glycérate déshydrogénase, 9 : glycérate kinase. Les étapes du cycle photorespiratoire sont représentées en bleu, celles du cycle de Calvin en rouge.

glycérate est alors phosphorylé par la glycérate kinase dans le chloroplaste, et le phosphoglycérate qui en résulte est converti en RuBP par le cycle de Calvin. Les réactions chimiques de la voie photorespiratoire impliquent une coordination étroite entre les métabolismes des chloroplastes, des mitochondries et des peroxisomes, organites souvent proches les uns aux autres dans les cellules photosynthétiques des végétaux supérieurs (Figure 9).

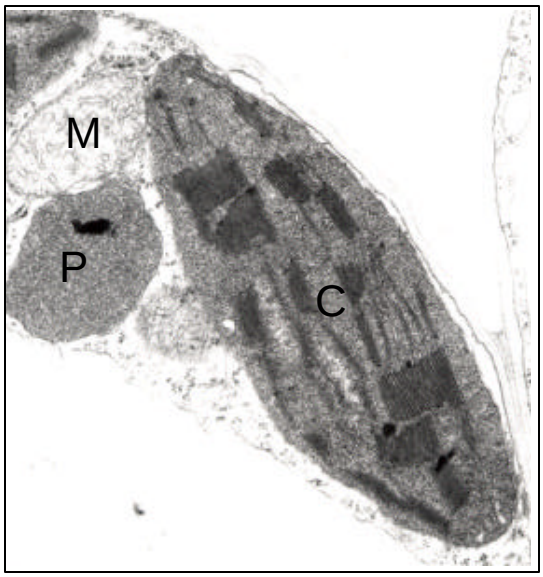

Figure 9. Micrographie par transmission électronique d'une cellule de *Nicotiana tabacum* (5000x) soulignant la promiscuité entre chloroplaste (C), mitochondrie (M) et peroxysome (P). Cliché aimablement fourni par F. Eymery (CEA Cadarache).

Bien que l'affinité de la Rubisco pour le CO_2 soit nettement plus forte que pour l'O_2, la forte teneur en oxygène de l'atmosphère (21%) comparée au CO_2 (0,035%), rend cette réaction non négligeable chez les végétaux supérieurs. En conditions naturelles, le taux de rejet de CO_2 par la photorespiration avoisinerait 25% à 50% du taux net d'assimilation du CO_2 (Gerbaud et André, 1979 ; Husic et al., 1987 ; Sharkey, 1988). La photorespiration est favorisée en situation de stress hydrique, la fermeture stomatique provoquant une baisse de la teneur interne en CO_2 et favorisant la photorespiration. Cette dernière aboutit à une perte d'énergie et de carbone mais pourrait jouer un rôle physiologique important en drainant l'excès de pouvoir réducteur, lorsque l'apport en CO_2 est limitant, ce qui permettrait d'éviter la formation de formes toxiques de l'oxygène et limiterait les dommages subis par l'appareil photosynthétique (Kozaki et Takeba, 1996).

La photorespiration augmente le coût énergétique de la fixation du CO_2. D'une part, le recyclage du NH_3 et la conversion du glycérate en 3-PGA sont des étapes qui nécessitent un apport énergétique sous forme d'ATP (Osmond, 1981). D'autre part, la conversion de la molécule de 3-phosphoglycerate issue du cycle photorespiratoire en RuBP, molécule disponible pour le cycle de Calvin, nécessite également de l'ATP. Ainsi, les rendements maximum de la photosynthèse sont obtenus en l'absence de photorespiration, comme par exemple sous de faibles teneurs en O_2 ou de fortes concentrations en CO_2 (CO_2 350 ppm ; O_2 1%). Dans ces conditions, la fixation d'une molécule de CO_2 nécessite 3 molécules d'ATP et 2 de NADPH ce qui correspond à un rapport ATP/NADPH de 1,5. Les coûts maximum de

fixation de CO_2, sont obtenus au point de compensation en CO_2, point pour lequel le bilan des échanges gazeux est nul, les réactions produisant du CO_2 (respiration et photorespiration) égalant les réactions consommant du CO_2 (photosynthèse). Dans ces conditions, la fixation d'une molécule de CO_2 nécessite 10 molécules d'ATP et 6 de NADPH, correspondant à un rapport ATP/NADPH de 1,66. L'assimilation du carbone requiert donc un ratio ATP/NADPH variant entre 1,5 et 1,66 selon l'activité photorespiratoire tandis que le transfert linéaire d'électrons permet la génération d'un ratio ATP/NADPH théorique variant entre 1 et 1,5 en fonction de l'efficience du cycle Q. Dans ces conditions, et même si l'on considère que le cycle Q est constitutif, le transfert linéaire d'électrons selon le schéma en « Z » est insuffisant pour subvenir aux besoins énergétiques de la fixation du carbone.

H. Respiration et photosynthèse

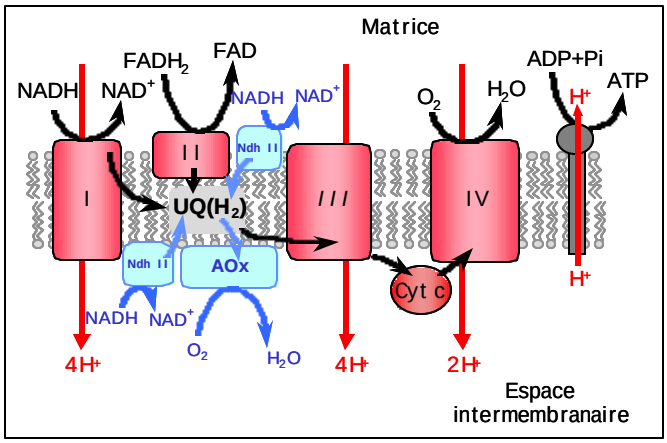

Figure 10. Représentation schématique du transfert d'électrons et des translocations de protons au sein de la membrane interne des mitochondries au cours de la respiration des plantes supérieures (d'après Vanlerberghe et McIntosh, 1997). Les transfert d'électrons sont représentés en noir, les translocations de protons en rouge. les complexes protéiques communs à tous les organismes eucaryotes sont représentés en rouge, les éléments alternatifs, propres aux organismes végétaux, en bleu. $UQ(H_2)$: ubiquinone/(ubiquinols), Complexe I : NADH déshydrogénase, complexe II : succinate déshydrogénase, complexe III : cytochrome bc_1 oxydoréductase, complexe IV : cytochrome *c* oxydase, Ndh II : NADH déshydrogénase de type II non électrogénique, AOx : oxydase alternative.

Dans la matrice des mitochondries, le cycle de Krebs réalise l'oxydation complète des molécules organiques en CO_2, aboutissant à la réduction du NAD^+ et du FAD. Le pouvoir réducteur ainsi formé est réoxydé par une chaîne de transporteurs d'électrons, faisant intervenir des complexes protéiques localisés au sein de la membrane interne des mitochondries. La première étape de ce transfert est assurée par le complexe NADH déshydrogénase (complexe I) qui oxyde le NADH (Figure 10). Les électrons transitent ensuite à travers le pool d'ubiquinone, le cytochrome bc_1 (complexe III) et le complexe cytochrome c oxydase (complexe IV). Au cours de ce transfert respiratoire d'électrons un gradient de protons est généré entre la

matrice et l'espace intermembranaire grâce à l'activité électrogénique des complexes I , III et IV, aboutissant à la synthèse d'ATP, via les ATPases qui utilisent la force proton-motrice ainsi générée. Les mitochondries végétales présentent également des voies de transferts alternatifs d'électrons (Douce et Neuburger, 1989) dont plusieurs types de NAD(P)H-ubiquinone réductases non électrogéniques qui permettent de réduire le pool d'ubiquinone à partir du NADH. Elles contiennent aussi une oxydase alternative (Vanlerberghe et McIntosh, 1997) qui permet de court-circuiter le transfert des électrons du pool d'ubiquinone directement vers l'oxygène moléculaire.

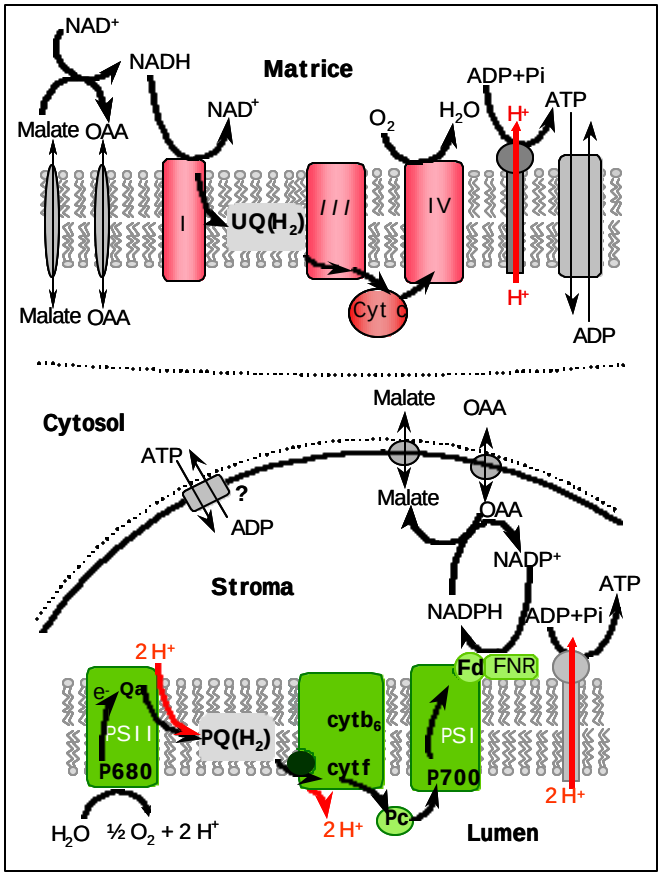

Figure 11. Schéma représentant certaines interactions rédox et énergétiques entre mitochondrie et chloroplaste (d'après Hoefnagel et al., 1998). La membrane externe du chloroplaste et de la mitochondrie, perméable, est représentée en pointillés. L'activité d'import du translocateur ATP/ADP de l'enveloppe chloroplastique est faible sur des chloroplastes matures. OAA : oxaloacetate.

Chez les végétaux supérieurs, la respiration et la photosynthèse ayant des bilans d'échanges gazeux antagonistes, il est difficile d'avoir une mesure précise de l'activité respiratoire lorsque la photosynthèse est active. La non-linéarité du rendement photosynthétique observée à faibles intensités lumineuses (appellée « effet Kok ») a été interprétée par une inhibition progressive de la respiration à la lumière (Kok, 1949 ; Sharp et al., 1984). La mesure de l'activité respiratoire à la lumière par l'incorporation de molécules radiomarquées intermédiaires du cycle du citrate a montré que celui-ci ainsi que la glycolyse sont des processus actifs à la lumière, même si leurs niveaux respectifs sont réduits (Graham, 1980). D'autres auteurs ont proposé que la respiration ne soit pas (Peltier et Thibault, 1985 ; Weger et al., 1989) ou seulement partiellement inhibée (Bate et al., 1988 ; Canvin et al., 1980) à la lumière.

Malgré l'absence de transporteurs spécifiques, le NADH peut être transporté grâce à l'activité de certains antiports tel que celui de l'oxaloacetate/malate (Heineke et al., 1991 ; Flügge, 1998). Ainsi, grâce à cet échange de métabolite et de pouvoir réducteur, les chloroplastes et les mitochondries sont en étroite relation avec le cytosol, ce qui permet un équilibre rédox entre compartiments (Krömer, 1995 ; Hoefnagel, 1998) (Figure 11). Chez *Chlamydomonas reinhardtii*, lorsque la respiration mitochondriale est inhibée avec de l'antimycine A et du SHAM à l'obscurité, le rapport NADPH/$NADP^+$ augmente dans les chloroplastes (Gans et Rebeillé, 1990) (Figure 11). Chez les végétaux supérieurs, Krömer et al. (1988) et Krömer et Heldt (1991) ont observé que l'oligomycine, un inhibiteur puissant des ATPases mitochondriales, réduisait les capacités photosynthétiques mesurées sur des protoplastes alors que celle-ci était inefficace sur des chloroplastes isolés. Ces auteurs proposent que les chloroplastes, excédentaires en NADPH (par rapport à l'ATP), exportent du pouvoir réducteur vers les mitochondries qui effectueraient sa conversion en ATP. Ce mécanisme participerait ainsi au ré-équilibrage du ratio ATP/NADPH au cours de la photosynthèse. Un mécanisme similaire a été proposé pour expliquer la croissance photoautotrophe de souches révertantes de *Chlamydomonas* dont l'ATPase chloroplastique est inopérationnelle (Lemaire et al., 1988). L'ATP pourrait être importé au sein du chloroplaste via le translocateur ATP/ADP ou à travers la navette dihydroxyacetone phosphate/ 3-PGA (Hoefnagel et al., 1998).

II. Transferts d'électrons alternatifs au schéma en « Z »

En dehors des réactions de transfert linéaire d'électrons impliquées dans le schéma classique du trajet en « Z » de la photosynthèse, d'autres voies de transfert d'électrons ont été décrites dans les chloroplastes des algues unicellulaires et des plantes supérieures.

A. Réaction de Mehler

Au cours de la photosynthèse, l'oxygène moléculaire peut être utilisé par le PS I comme accepteur final d'électrons de la chaîne photosynthétique , conduisant à la formation d'anion superoxyde au cours de réactions appelées réactions de Mehler (Mehler, 1951 ; Mehler et

Brown, 1952). Les formes actives de l'oxygène ainsi générées sont détoxifiées par une suite de réactions enzymatiques faisant intervenir la superoxyde dismutase (SOD) et le cycle ascorbate-glutathion. Les radicaux superoxyde sont transformés en peroxyde d'hydrogène (H_2O_2) par la SOD. Le H_2O_2 est ensuite réduit en H_2O par l'ascorbate peroxydase (APx). La régénération de l'ascorbate se fait soit via la ferrédoxine et une monodéhydroascorbate réductase NADP-dépendante, soit par l'intermédiare d'une déhydroascorbate réductase et de la glutathion réductase (Foyer et Haliwell, 1976 ; Miyake et Asada, 1994). Cette séquence de réactions enzymatiques qui produit de l'oxygène au niveau du PS II et en consomme au niveau du PS I conduit à un bilan nul pour les échanges nets d'O_2. Cependant, ce transfert d'électrons permet l'établissement d'un gradient de protons et la synthèse d'ATP. Pour cette raison, l'ensemble de ces réactions est également appelé photophosphorylation pseudocyclique (Asada, 1999) (Figure 12).

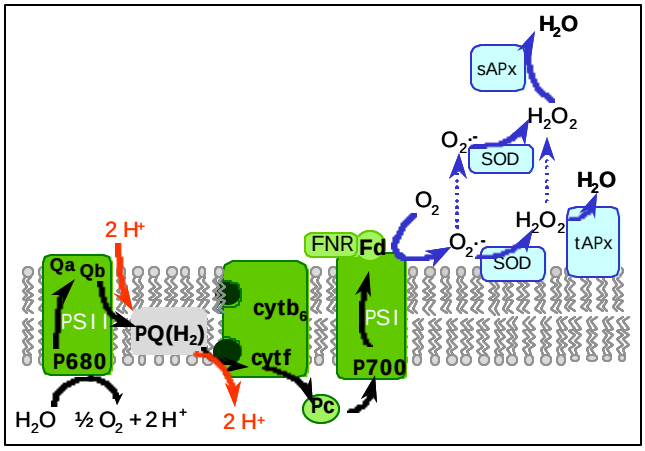

Figure 12. Schéma représentant le transfert pseudocyclique d'électrons et les réactions de photoréduction directe de l'oxygène (d'après Asada, 1999). Les transferts d'électrons linéaires sont représentés en noir, les translocations de protons en rouge. Les transfert alternatifs correspondant à la détoxication des formes actives de l'oxygène sont représentés en bleu. SOD : superoxyde dismutase, tAPx : ascorbate peroxydase ancrée dans la membrane des thylacoïdes, sAPx : ascorbate peroxydase du stroma.

Les réactions de Mehler ont été mises en évidence sur des chloroplastes isolés (Mehler, 1951; Asada, 1999), mais leur importance réelle au sein des feuilles intactes reste contreversée (Badger, 1985; Badger et al., 2000). Chez les plantes C_3, des données intéressantes proviennent de l'utilisation de plantes de tabac transgéniques antisens pour l'ARNm de la petite sous-unité de la Rubisco. Chez des plantes dont la teneur en Rubisco est réduite de 90%, la relation entre le transport des électrons et l'assimilation du CO_2 reste parfaitement linéaire quelle que soit l'intensité lumineuse (Ruuska et al., 2000). La baisse des activités carboxylase et oxygénase de la Rubisco n'entraîne pas une réorientation des électrons vers d'autres accepteurs terminaux d'électrons tels que l'O_2 au cours des réactions de Mehler (Ruuska et al., 2000; Badger, 2000). Il a toutefois été proposé que le transfert pseudocyclique intervienne lors d'un stress hydrique (Asada, 1999 ; Polle, 1996 ; Flexas et al., 1999), et puisse intervenir dans la dissipation de l'énergie lumineuse, minimisant les

dommages que pourraient occasionner cette situation (Asada, 2000). Cependant, d'après Badger et al., (2000) , les réactions de Mehler ne semblent pas représenter un flux important d'électrons chez les plantes C_3, la photorespiration étant vraisemblablement responsable de la grande majorité de la prise d'O_2 mesurée à la lumière.

B. Transfert cyclique des électrons autour du PS I

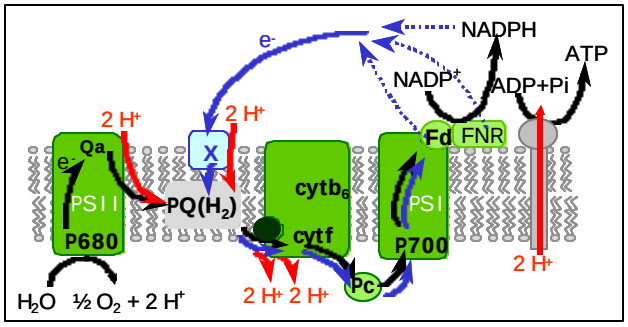

Figure 13. Schéma représentant le transfert cyclique des électrons autour du PS I. Les transferts d'électrons linéaires sont représentés en noir, les translocations de protons en rouge. Les transferts alternatifs d'électrons reliés au transfert cyclique autour du PS I sont représentés en bleu.

En l'absence de PS II, une synthèse d'ATP est possible grâce à un transfert cyclique d'électrons autour du PS I au cours duquel les électrons situés coté accepteurs du PSI (ferrédoxine, FNR ou NADPH) sont redirigés vers les transporteurs situés entre les deux photosystèmes, tels que le pool de PQ ou le cytochrome b_6/f (Huber et Edwards, 1976 ; Malkin et Chain, 1980). Ce mécanisme s'accompagne d'un transfert de protons du stroma vers le lumen des thylacoïdes permettant ainsi l'établissement de la synthèse d'ATP par le jeu des photophosphorylations cycliques (Arnon, 1955). Initiallement, ces mécanismes ont été étudiés *in vitro* en suivant l'ATP formé sur des thylacoïdes isolés (Arnon et Chain, 1975 ; Bedell et Govindjee, 1973) ou les variations de pH du lumen sur des chloroplastes intacts avec un traceur fluorescent tel que l'aminoacridine (Heber et al., 1978). Sur des thylacoïdes isolés, ces réactions nécessitent généralement l'ajout de ferrédoxine réduite (Arnon et al., 1967 ; Arnon et Chain, 1975 ; Malkin et Chain, 1980 ; Moss et Bendall, 1984). Le transfert cyclique des électrons autour du PS I, de la même manière que le transfert linéaire des électrons, est inhibé par le DBMIB, un analogue des quinones qui agit au niveau du site Q_o du cytochrome b_6/f (Trebst, 1974; Cleland et Bendall, 1992). L'oxydation du pool de PQ par le complexe cytochrome b_6/f apparaît donc comme une étape obligatoire du transfert cyclique, le retour des électrons en provenance du côté accepteur du PS I se faisant préférentiellement au niveau du pool de PQ (Figure 13).

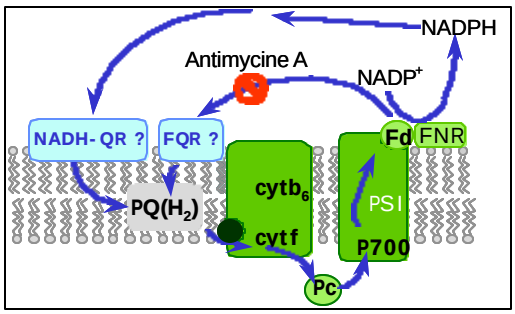

Figure 14. Représentation schématique des différentes voies de transfert cyclique des électrons autour du PS I proposées *in vitro* sur des chloroplastes ou *in vivo* chez l'algue unicellulaire *Chlamydomonas reinhardtii* (d'après Ravenel et al., 1994). FQR : ferrédoxine PQ oxydoréductase ; NADH-QR : NADH PQ oxidoréductase.

Le transfert cyclique est inhibé par l'antimycine A (Tagawa et al., 1963 ; Arnon et al., 1967) alors que cet antibiotique n'affecte ni le cytochrome b_6/f ni le transfert linéaire (Hauska et al., 1983). La nature moléculaire des transporteurs d'électrons impliqués dans le transfert cyclique et sensibles à l'antimycine n'est pas élucidée. La ferrédoxine réduite étant capable de réduire la chaîne de transfert d'électrons (Tagawa et al., 1963 ; Arnon et Chain, 1979 ; Kimata-Ariga et al., 2000) et il a été suggéré qu'une activité ferrédoxine plastoquinone réductase (FQR) soit impliquée dans la réduction du pool de PQ (Moss et Bendall, 1984). Cette enzyme, qui n'est caractérisée ni au niveau biochimique ni au niveau moléculaire, pourrait constituer le site d'action de l'antimycine A (Cleland et Bendall, 1992). Etant donné le fort pouvoir inhibiteur de l'antimycine A sur le transfert cyclique des électrons, Bendall et Manasse (1995) ont suggéré que la voie de réduction impliquant la FQR pourrait constituer la voie principale de transfert cyclique. Cependant, différents auteurs ont suggéré l'existence d'autres voies de transfert cyclique autour du PSI (Figure 14). Ainsi, Hosler et Yocum (1985) ont décrit des conditions expérimentales au cours desquelles le transfert cyclique étudié sur des thylacoïdes isolés était insensible à l'antimycine A et ont proposé que, sous certaines conditions, une autre voie de réduction du pool de PQ puisse prendre en charge l'activité cyclique. La FNR pourrait être impliquée dans la voie insensible à l'antimycine A (Hosler et Yocum, 1985 ; Cleland et Bendall, 1992 ; Scheller, 1996). D'autres voies de réduction non photochimique des plastoquinones, impliquant notamment des activités de type NAD(P)H PQ oxydoréductases, ont été décrites. Ainsi, sur des membranes de thylacoïdes de *Chlamydomonas reinhardtii*, le NADH peut céder ses électrons à la chaîne photosynthétique (Godde et Trebst, 1980). De plus, un apport exogène de NADH ou de NADPH sur des thylacoïdes d'épinards ou de pomme de terre permet la réduction non photochimique des PQ (Mills et al., 1979 ; Endo et al., 1997; Corneille et al., 1998). L'existence de plusieurs voies de transfert cyclique des électrons autour du PS I pourrait conférer une certaine flexibilité à ce mécanisme. Ainsi, Hosler et Yocum (1987) ont conclu que la contribution respective des deux voies dépendait des conditions rédox. L'existence de deux voies de transfert cyclique, dont l'une sensible à

l'antimycine A, a été également proposée *in vivo*, chez *Chlamydomonas reinhardtii* (Ravenel et al., 1994).

Le transfert cyclique des électrons, qui permet une synthèse d'ATP sans générer de pouvoir réducteur, pourrait participer au ré-équilibrage du ratio ATP/NADPH au sein des chloroplastes et ainsi permettre l'optimisation du cycle de Calvin. L'effet inhibiteur de l'antimycine A sur la fixation du CO_2 mesurée sur des chloroplastes (Woo, 1983) ou sur des protoplastes (Furbank et Horton, 1987) a été interprété par une participation des photophosphorylations cycliques à l'assimilation du CO_2.

In vivo, les variations d'absorption à 545 nm sont correlées aux variations du gradient de pH entre le lumen et le stroma (Heber, 1978) et ont été utilisées pour évaluer l'activité du transfert cyclique des électrons autour du PSI. Par ailleurs, des mesures de fluorescence de la chlorophylle, soit en conditions anaérobies (Harris et Heber, 1993), soit après une période d'éclairement ont été utilisées pour mettre en évidence la réduction non-photochimique du pool de PQ (Groom et al., 1993 ; Feild et al., 1998 ; Farineau, 2000). De même, des mesure de cinétique de re-réduction du P_{700}^{+}, après son oxydation avec un éclairement en rouge lointain, permettent d'estimer l'entrée des électrons dans la chaîne de transfert d'électrons à partir du pouvoir réducteur stromal (Schreiber et al., 1988). Ces mesures ont été considérées par certains auteurs comme rendant compte de l'activité cyclique autour du PS I (Schreiber et al., 1988 ; Asada et al., 1993).

La technique de spectrométrie photoacoustique permet une estimation de l'activité cyclique par la mesure du stockage photochimique d'énergie. Les variations d'émission de chaleur en réponse à un éclairement modulé, modifient la pression de l'air de la couche limite entourant l'échantillon foliaire et forment une onde acoustique mesurée à l'aide d'un microphone (Lasser-Ross et al., 1980). L'excitation sélective du PS I par un éclairement rouge lointain permet de mesurer le stockage photochimique qui rend compte de l'activité de transfert cyclique. Par cette technique, différents auteurs ont souligné les très faibles activités de transfert cyclique mesurées chez les plantes C_3 (Herbert et al., 1990; Malkin et al., 1992 ; Havaux et al., 1991 ; Havaux, 1992) si on les compare aux plantes C_4 (Herbert et al., 1990 ; Havaux, 1992 ; Asada et al., 1993), aux algues unicellulaires (Maxwell et Biggins, 1976; Ravenel et al., 1994) ou aux cyanobactéries (Mi et al., 1992). Au sein des cellules de la gaine périvasculaire des plantes C_4, le PS II est très peu abondant (Woo et al., 1970) et l'assimilation du carbone est dépendante de l'activité du transfert cyclique autour du PSI pour la fourniture en ATP (Legood et al., 1981). Chez les cyanobactéries, il a été suggéré que l'activité de transfert cyclique serve à l'énergisation de la membrane permettant l'import de

certains ions tel que bicarbonate (pour une revue, voir Fork et Herbert, 1993) et soit stimulée lors de stress salin (Jeanjean et al., 1993).

Il est généralement considéré que l'activité cyclique des plantes C_3 est faible et qu'elle joue un rôle mineur dans l'assimilation du CO_2 (Bendall et Manasse, 1995 ; Fork et Herbert, 1993). L'activité cyclique pourrait être toutefois stimulée en conditions de stress et permettre une protection du PSII grâce à un contrôle de son activité par le pH du lumen (Heber et Walker, 1992 ; Heber et al., 1995 ; Cornic et al., 2000). Certains auteurs ont proposé que l'activité de photophosphorylation cyclique soit régulée par l'état rédox de la chaîne de transfert d'électrons (Arnon et Chain, 1975, 1979) ou au niveau du pool de NADPH (Takahama et Heber, 1981 ; Hosler et Yocum, 1987). L'activité du transfert cyclique serait inhibée lorsque la chaîne de transfert d'électrons est totalement réduite (Ziem-Hanck et Heber, 1980) ou totalement oxydée (Arnon et Chain, 1979). Il a été proposé que l'oxygène moléculaire intervienne dans œs processus en interagissant au niveau des accepteurs du PSI (Arnon et Chain, 1975 ; Heber et al., 1978) ou entre les deux photosystèmes (Harris et Heber 1993).

Bien que les études menées *in vitro* aient permis de caractériser plusieurs voies de transfert cyclique autour du PSI, la nature moléculaire des transporteurs d'électrons impliqués dans la réduction du pool de PQ reste à déterminer. *In vivo,* l'existence d'un transfert cyclique chez les plantes de type C_3 ainsi que le rôle physiologique de ce processus restent à établir

C. Chlororespiration

L'existence d'une interaction entre le pool de PQ et l'oxygène moléculaire a été proposée pour expliquer les effets de l'anaérobiose (Diner et Mauzerall, 1973) ou d'inhibiteurs d'oxydases respiratoires (Bennoun, 1982) sur l'état rédox du pool de PQ à l'obscurité chez des algues unicellulaires telles que *Chlamydomonas reinhardtii*. Le terme de « chlororespiration » a été utilisé pour décrire l'activité d'une chaîne de transfert d'électrons respiratoire hypothétique qui existerait au sein de la membrane thylacoïdienne des chloroplastes (Bennoun, 1982). D'après le modèle initialement proposé, la chlororespiration ferait intervenir une étape de réduction non photochimique du pool de PQ et une étape d'oxydation non photochimique du pool de PQ utilisant l'oxygène moléculaire comme accepteur final d'électrons (Figure 15). En parallèle, un gradient électrochimique serait généré de part et d'autre de la membrane thylacoïdienne. Chez les plantes supérieures, certains auteurs ont rapporté les effets d'inhibiteurs d'oxydases respiratoires, tels que le cyanure et le monoxyde de carbone, sur l'état rédox des transporteurs d'électrons entre les deux

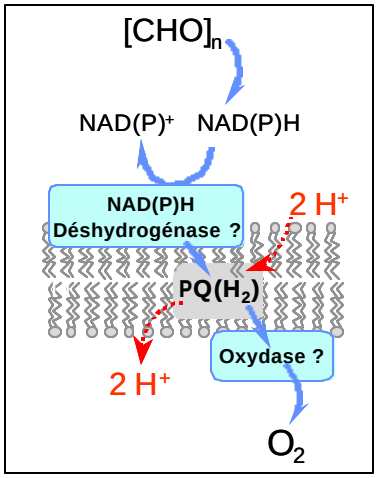

Figure 15. Représentation schématique des interactions possibles entre le pool de PQ et une chaîne respiratoire hypothétique au sein des thylacoïdes (d'après Bennoun, 1982).

photosystèmes, et ont conclu à l'existence de la chlororespiration (Garab et al. 1989 ; Feild et al. 1998). Toutefois, l'interprétation de l'ensemble de ces expériences, réalisées sur des organismes intacts, était basée sur l'hypothèse implicite que le chloroplaste et la mitochondrie ne sont pas en interaction rédox. La découverte d'une communication rédox entre les deux organites (voir paragraphe I.H) permettrait d'expliquer ces effets par une inhibition de la respiration mitochondriale. Ainsi, il a été rapporté que l'inhibition de la respiration mitochondriale chez *Chlamydomonas reinhardtii* aboutit à une augmentation de la concentration en NADPH dans le stroma des chloroplastes et favoriserait la réduction non photochimique du pool de PQ (Gans et Rebeillé, 1990). L'utilisation de souches de *Chlamydomonas reinardhtii* mutantes dont le cytochrome bc_1 mitochondrial est insensible au myxothiazol a permis de démontrer que l'effet de ce composé sur le niveau d'oxydation des PQ est lié à une inhibition de la respiration mitochondriale (Bennoun, 1994).

Il a été montré, par ailleurs, que des flashs lumineux inhibent transitoirement une prise d'oxygène de type respiratoire chez *Chlamydomonas reinhardtii* (Peltier et al., 1987). Ce phénomène étant insensible à l'inhibition de la respiration mitochondriale, il a été attribué à une interaction électronique entre chaînes de transfert d'électrons photosynthétique et chlororespiratoire. Ces auteurs ont observé que le PSI inhibe transitoirement l'activité chlorespiratoire, tandis que le PSII la stimule.

Après sa réduction, notamment en réponse à une forte illumination, le pool de PQ est réoxydé par une voie non photochimique qui fait intervenir l'O_2 (Harris et Heber 1993). Il a été proposé que le cytochrome b_{559} (Kruk et Strzalka, 1999) ou une hydroquinone peroxydase (Casano et al., 2000) soient impliqués dans ce processus.

Différentes fonctions ont été attribuées à la chlororespiration parmi lesquelles la production d'ATP et l'énergétisation de la membrane thylacoïdienne à l'obscurité (Bennoun, 1982), la réoxydation du pouvoir réducteur généré par la dégradation de l'amidon (Gfeller et Gibbs, 1985; Singh et al., 1992) ou encore l'adaptation à des conditions environnementales extrêmes (Peltier et Schmitt, 1991; Lajko et al., 1997). Cependant, en l'absence d'identification d'une oxydase chloroplastique, l'existence d'une activité chlororespiratoire a été largement controversée (Bennoun, 1994 ; Bennoun, 1998; Nixon, 2000).

III. Bases moléculaires des voies alternatives de transferts d'électrons

A. Découverte d'un complexe NAD(P)H déshydrogénase chloroplastique

L'analyse du génome chloroplastique de plantes aussi différentes que l'hépatique (Ohyama et al., 1986), le tabac (Shinozaki et al., 1986) ou le riz (Hiratsuka et al., 1989) ont révélé l'existence de gènes présentant de fortes homologies avec les gènes mitochondriaux codant pour les sous-unités du complexe NADH ubiquinone oxydo-réductase mitochondrial (complexe I). Dans les génomes chloroplastiques séquencés, onze des quatorze gènes codant pour les sous-unités du complexe I bactérien ont été identifiés. Le complexe I bactérien est décrit comme étant le complexe « minimal ». Il ne comprend que quatorze sous-unités alors qu'au moins 41 polypeptides différents ont été mis en évidence pour le complexe I mitochondrial de bœuf (Arizmendi et al., 1992). Les 3 « gènes manquants » des génomes chloroplastiques correspondent à ceux codant pour les sous-unités du domaine catalytique de liaison au NADH (Friedrich et al., 1995) (Figure 16). Les gènes *ndh* sont transcrits (Matsubayashi et al., 1987 ; Kanno et Hirai, 1993), certains des ARNm sont édités (Freyer et al., 1995 ; Maier et al., 1995) et traduits. Les sous-unités du complexe Ndh ont été identifiées au sein des membranes thylacoïdiennes (Nixon et al., 1989 ; Berger et al., 1993 ; Kubicki et al., 1996). Récemment, un complexe présentant une activité de type NADH-déshydrogénase et contenant les produits de certains gènes *ndh*, a été purifié à partir de membranes de thylacoïdes de pois (Sazanov et al. 1998) et d'orge (Quiles et al., 2000).

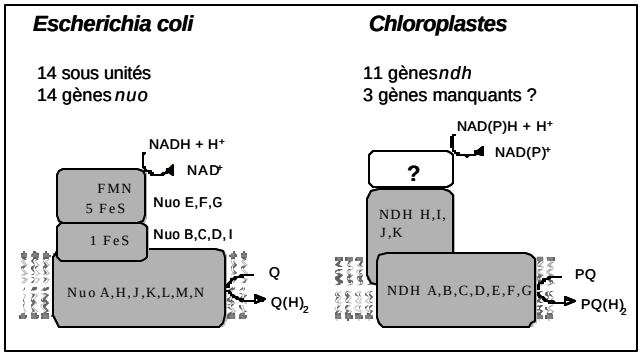

Figure 16. Modèle structural du complexe I bacterien et représentation hypothétique du complexe Ndh chloroplastique (d'après Friedrich, 1995). Les gènes codant pour les sous-unités du domaine catalytique de liaison au NADH ne sont pas connu pour le complexe Ndh chloroplastique. FMN : flavine mono nucléotide.

Certaines études ont suggéré une association entre le complexe Ndh et la FNR, cette dernière pouvant constituer le domaine catalytique de liaison au NADPH du complexe Ndh (Guedeney et al., 1996 ; Quiles et Cuello, 1998). Cependant, cette hypothèse n'est pas en accord avec la spécificité pour le NADH du complexe purifié (Sazanov et al., 1998 ; Casano et al., 2000). De par sa fonction supposée, le complexe Ndh pourrait donc jouer un rôle dans les phénomènes de réduction non photochimique du pool de PQ et participer au transfert cyclique des électrons autour du PS I ou à la chlororespiration.

L'absence des gènes *ndh* du génome chloroplastique de plantes parasites telles qu'*Epifagus virginia* (dePampihilis et Palmer, 1990 ; Wolfe et al ; 1992), qui ont également perdu les gènes codant pour des fonctions photosynthétiques, témoignerait d'un lien fonctionnel entre les produits de ces gènes. Cependant, l'absence de gènes *ndh* chez *Chlamydomonas reinhardtii* et chez *Pinus thunbergii* (Wakasugi et al., 1994) pose la question de l'importance physiologique réelle du complexe Ndh *in vivo*.

L'hypothèse d'une implication du complexe Ndh dans le transfert cyclique des électrons autour du PS I est compatible avec sa localisation membranaire (Nixon et al., 1989; Berger et al., 1993). Le complexe Ndh (Burrows et al. 1998) a été localisé dans les membranes lamellaires des thylacoïdes, où le PS I est majoritaire par rapport au PS II (Berger et al., 1993). De plus, la forte représentation de sous-unités Ndh dans les cellules de la gaine périvasculaire des plantes C_4 est corrélée à une activité de transfert cyclique importante (Kubicki et al., 1996). Cependant, le rôle du complexe Ndh dans le transfert cyclique des électrons et dans la synthèse d'ATP au cours des réactions photochimiques n'a pas pu être prouvé chez les plantes supérieures.

Chez les cyanobactéries, l'inactivation des gènes *ndhB* ou *ndhL* chez *Synechocystis* (Ogawa, 1991) et chez *Synechococcus* (Marco et al., 1993) affecte le transport du carbone inorganique et la croissance n'est permise qu'à de fortes concentrations en CO_2. Il a été proposé que le complexe Ndh cyanobactérien participe au transfert cyclique d'électrons et maintienne ainsi le gradient électrochimique, nécessaire à l'energisation du système de transport du carbone inorganique (Ogawa, 1991). Des mesures de photoacoustique et de re-réduction du P_{700}^+ montrent que les processus de transfert cyclique sont fortement affectés chez les mutants de cyanobactéries déficients en certaines sous-unités Ndh (Mi et al., 1995). Ce mécanisme est insensible à l'antimycine A et inhibé par un inhibiteur du complexe I, la roténone (Mi et al., 1992, 1995).

B. Découverte d'une oxydase terminale chloroplastique

Une protéine homologue aux oxydases alternatives mitochondriales de type quinol oxydase a été récemment découverte dans les chloroplastes, (Wu *et al.*, 1999 ; Carol *et al.*, 1999) (Figure 17). Cette protéine (appelée PTOX, pour Plastid Terminal OXidase) est impliquée dans la synthèse des caroténoides, notamment au cours de la biogénèse des chloroplastes. En effet, les mutants d'*Arabidopsis thaliana* dépourvus de cette protéine exhibent des secteurs dépourvus de pigments. Ces mutants s'avèrent incapables d'effectuer la désaturation du phytoène, une étape qui nécessite les plastoquinones oxydées comme accepteur d'électrons (Wetzel et al., 1994 ; Norris et al., 1995). L'absence de PTOX conduirait à une réduction importante du pool de PQ et donc à une capacité réduite à synthétiser des caroténoïdes, causant des dommages photo-oxydants et la destruction des pigments chlorophylliens (Wu *et al.*, 1999 ; Carol *et al.*, 1999).

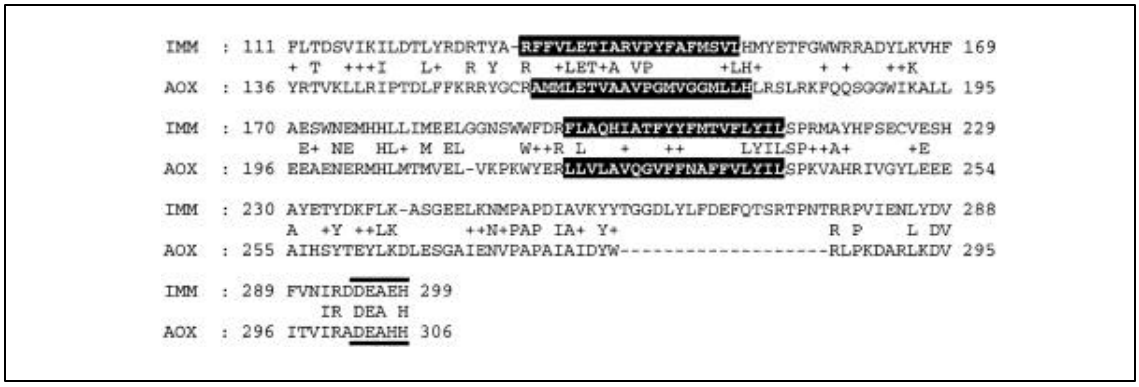

Figure 17. Homologies entre la protéine PTOX d'*Arabidopsis thaliana* et l'oxydase alternative mitochondriale de soja (d'après Carol et al., 2000). Les acides aminés identiques sont identifiés par leur lettre tandis que les acides aminés similaires sont identifiés par un signe +. Le cadre noir correspond à un domaine prédit pour être une hélice transmembranaire. Un domaine potentiel de liaison à l'atome de fer est souligné.

Au sein des mitochondries végétales, l'oxydase alternative, quant-à-elle, permet de court-circuiter le transfert des électrons du pool d'ubiquinone directement vers l'oxygène moléculaire, éliminant ainsi deux étapes électrogéniques (l'ubiquinone cytochrome C réductase et la cytochrome C oxydase). Ce mécanisme de valve rédox permettrait d'éviter l'engorgement de la chaîne de transfert d'électrons (Vanlerberghe et McIntosh, 1997) limitant ainsi la formation de formes actives de l'oxygène. Des cultures cellulaires de tabac transgéniques qui surexpriment cette protéine génèrent moins de formes actives de l'oxygène

que des cultures cellulaires issue de plantes de type sauvage (Maxwell et al., 1999). De plus, l'expression de l'oxydase alternative mitochondriale est stimulée dans des conditions décrites pour générer un stress oxydant et la formation d'espèces actives de l'oxygène, comme par exemple un stress thermique, la senescence ou les attaques d'organismes pathogènes (Vanlerberghe et McIntosh, 1997).

Comme pour les oxydases alternatives de type mitochondrial, PTOX est insensible au cyanure, au CO et à l'azide de sodium (Cournac et al., 2000) tandis qu'elle est inhibée par le propyl gallate (Josse et al., 2000). PTOX, lorsqu'elle est exprimée de manière hétérologue dans *Escherichia coli,* confère aux membranes des bactéries une activité respiratoire liée à une quinol oxydase sensible au propyl gallate. Cependant son rôle *in vivo* dans les mécanismes d'oxydation non photochimique du pool de PQ, dans la chlororespiration ou dans les processus de valve rédox en situation d'engorgement de la chaîne photosynthétique de transfert d'électrons reste à établir. Chez des souches de *Chlamydomonas reinhardtii* déficientes en PSI, le propyl gallate inhibe le pic transitoire de prise d'oxygène initié par un flash lumineux lié à l'activité du PS II (Cournac et al., 2000). Il a été proposé qu'une protéine homologue à PTOX soit impliquée dans l'oxydation non photochimique du pool de PQ (Cournac et al., 2000).

Objectifs de thèse

Bien que le transfert cyclique des électrons autour du PS I ait été largement étudié *in vitro*, sa participation *in vivo* aux mécanismes de la photosynthèse reste à démontrer, notamment chez les plantes supérieures de type C_3. Par ailleurs, l'existence d'une chlororespiration, initialement proposée pour rendre compte des effets d'inhibiteurs d'oxydases respiratoires sur l'état rédox des transporteurs photosynthétiques d'électrons, est quant-à-elle fortement contreversée. Dans un cas comme dans l'autre, les transporteurs d'électrons susceptibles d'être impliqués dans ces processus n'ont été caractérisés ni au niveau biochimique, ni au niveau moléculaire.

De par ses homologies de séquence avec les complexes I mitochondriaux et bactériens, le complexe Ndh chloroplastique des végétaux supérieurs pourrait être impliqué dans la réduction du pool de PQ et ainsi participer au transfert cyclique des électrons autour du PS I et/ou à la chlororespiration. D'autre part, la protéine PTOX, homologue à l'alternative oxydase mitochondriale et impliquée dans la biosynthèse des caroténoïdes, semble être un candidat possible pour catalyser l'oxydation des plastoquinols.

Un des objectifs de cette thèse était de déterminer la fonctionalité du complexe Ndh et de la protéine PTOX dans les réactions de transfert d'électrons au sein des thylacoïdes. Pour ce faire, nous avons cherché à modifier l'expression des ces protéines chez *Nicotiana tabacum*, en utilisant les techniques de transgénèse végétale (transformation des génomes chloroplastiques et nucléaires) disponibles chez cette espèce. Des techniques biophysiques (fluorescence de la chlorophylle, fluorescence des pyridines nucléotides, changements d'absorption), ont été utilisées pour caractériser *in vivo* l'effet de ces modifications sur les réactions thylacoïdiennes de transfert d'électrons et déterminer le rôle de ces complexes dans la réduction et l'oxydation non-photochimique du pool de PQs. Par ailleurs, la participation du complex Ndh dans les réactions de transfert cyclique d'électrons autour du PS I a été recherchée par spectrométrie photoacoustique.

Enfin, dans le but de déterminer la fonction physiologique de ces transporteurs (Ndh et PTOX), nous avons cherché à déterminer dans quelle mesure les capacités d'assimilation photosynthétique étaient affectées chez les plantes mutantes obtenues. Un intérêt particulier a été porté aux conditions physiologiques, telles que le déficit hydrique, où la demande en ATP est accrue.

Chapitre I

Obtention et caractérisation de transformants chloroplastiques dépourvus de complexe Ndh: implication du complexe dans la réduction non photochimique du pool de PQ.

Le séquençage du génome chloroplastique de différentes plantes supérieures (Ohyama et al., 1986 ; Shinozaki et al., 1986 ; Hiratsuka et al., 1989) a révélé l'existence de onze gènes (gènes *ndh*) homologues aux gènes codant pour des sous-unités des complexe I mitochondriaux et bactériens (Friedrich et al., 1995). La démonstration de la fonctionnalité des gènes *ndh* par la mise en évidence des ARNm et polypeptides correspondants, a rapidement conduit à suggérer l'existence d'un complexe analogue au complexe I dans le chloroplaste.

Afin d'établir le rôle du complexe Ndh chloroplastique, nous avons adopté une approche visant à inactiver la biosynthèse d'une des sous-unités codées par un des gènes *ndh* porté par le plastome. Cette démarche expérimentale procède de la transformation chloroplastique, une technique relativement récente chez les végétaux supérieurs, et mise au point chez *Nicotiana tabacum* (Svab et al., 1990). Deux techniques de transformation ont été utilisées : celle de « biolistique », où des tissus foliaires sont bombardés avec des billes de tungstène sur lesquelles est fixé l'ADN vecteur (Svab et Maliga, 1990) et celle dite « au PEG (polyethylène glycol) » où des protoplastes en présence de l'ADN sont traités par une solution de PEG modifiant les membranes et favorisant l'entrée de l'ADN dans le chloroplaste (O'Neill, 1993). La transformation chloroplastique procédant de la recombinaison homologue, on utilise un plasmide porteur du gène d'intérêt et contenant de part et d'autre des régions flanquantes homologues à celles de la zone d'intégration. La sélection des plantes transplastomiques

s'effectue à l'aide de marqueurs de résistance, la pression de sélection permettant d'atteindre un état d'homoplasmie où toutes les copies du génome chloroplastique de l'ensemble des chloroplastes (de l'ensemble des cellules) sont recombinantes. Deux marqueurs de sélection sont couramment utilisés. Il s'agit soit du gène *aad*A dont le produit, l'aminoglycoside adényl transférase, détoxique la spectinomycine ou la streptomycine (Svab et Maliga, 1993), soit du gène *16SrDNA* présentant une mutation ponctuelle qui rend son produit l'ARN ribosomique 16S, insensible à la spectinomycine et/ou à la streptomycine (O'Neill et al.,1993). Le marqueur *aad*A est dit "dominant", et les transformants sélectionnés doivent subir plusieurs cycles différenciation/dédifférenciation (régénération) sur le milieu de sélection avant d'atteindre l'homoplasmie (O'Neill et al.,1993). Le marqueur alternatif *16SrDNA* est considéré comme un marqueur « récessif » et son utilisation permet d'atteindre immédiatement l'homoplasmie (O'Neill et al., 1993); cet avantage expérimental est limité car l'emploi de ce marqueur ne pourra être envisagé que pour des modifications affectant la zone proximale du gène *16SrDNA* sur le plastome. En collaboration avec le laboratoire de Peter Medgyesy (Biological Research Center, Szeged, Hongrie), qui avait mis au point cette technique de transformation, nous avons choisi d'inactiver le gène *ndh*B, qui n'est distant que de 3,6 kb du gène *16SrDNA*. Une construction contenant une partie du gène *ndh*B modifié par l'addition d'une paire de bases dans sa séquence provocant ainsi un décalage du cadre de lecture et engendrant plusieurs codons stop dès le début de la séquence et le gène *16SrDNA* portant la mutation conférant la résistance à la spectinomycine a été réalisée. Cette construction a été introduite dans des protoplastes par traitement au PEG. Après sélection, des transformants homoplasmiques ont été obtenus. La caractérisation biochimique et moléculaire des mutants (voir l'article joint Horvath et al. (2000) Plant Physiology, 123 : 1337-1349) a permis de montrer que le complexe Ndh est absent des membranes thylacoïdiennes. D'autre part, des mesures de fluorescence de la chlorophylle (voir l'article joint Cournac et al. (1998) G. Garab (ed.), Photosynthesis : Mechanisms and effects, Vol III, 1877-1882) ont permis de démontrer que le complexe Ndh, fonctionnel *in vivo*, est directement impliqué dans la réduction non photochimique du pool de PQ. Les plants de tabac mutants présentent des caractéristiques de croissance et des capacités photosynthétiques similaires aux plantes de type sauvage dans la plupart des conditions testées. Par contre, dans des conditions de stress hydrique modéré, un écart de croissance est observé entre les plantes sauvages et les plantes témoins. Nous avons fait l'hypothèse que cette différence phénotypique était observé dans des conditions où la disponibilité interne en CO_2 est faible et où une forte activité photorespiratoire augmente la demande métabolique en ATP. Le complexe Ndh serait

impliqué dans un mécanisme, tel que le transfert cyclique des électrons, capable de fournir de l'ATP supplémentaire.

Parallèlement à nos expériences, plusieurs laboratoires ont utilisé les techniques de transformation du génome chloroplastique en vue d'obtenir des plantes transplastomiques dont le complexe Ndh ne serait pas fonctionnel. Ainsi, Kofer et al. (1998) ont procédé à l'inactivation individuelle des gènes *ndh A,C,H,I,,J,K* appartenant à deux opérons polycistroniques contenant d'une part *ndh C,K,J* et d'autre part *ndh H,A,I,G,E, psaC, ndh D.* En utilisant la cassette de sélection aadA, ces auteurs ne sont pas parvenus à atteindre l'homoplasmie et ont conclu que le complexe Ndh était indispensable à la survie des plantes. Burrows et al. (1998) ont également procédé à l'inactivation individuelle de chaque gène de l'opéron *ndh C,K,J.* En utilisant la même cassette de sélection aadA, ces auteurs ont obenus l'homoplasmie et des mutants dont le complexe Ndh n'était pas fonctionnel. Ces auteurs concluent que le complexe Ndh n'est pas indispensable à la survie des plantes et proposent que Kofer et al. (1998) ont également atteint l'homoplasmie sans s'en rendre compte à cause de contaminations par des pseudogènes *ndh* nucléaires lors de l'analyse Southern (Maliga et Nixon, 1998). Enfin, Shikanai et al. (1998) ont inactivé le gène *ndhB* en utilisant la cassette de sélection aadA. Ces auteurs ont atteints l'homoplasmie et ont conclu, comme les autres équipes et la notre, à l'implication du complexe dans les mécanismes de réduction non photochimique du pool de PQ.

A. Targeted inactivation of the plastid *ndhB* gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure

Horvath et al. (2000) *Plant Physiology* **123**, 1337-1349.

Targeted Inactivation of the Plastid *ndhB* Gene in Tobacco Results in an Enhanced Sensitivity of Photosynthesis to Moderate Stomatal Closure[1]

Eva M. Horváth, Stefan O. Peter[2], Thierry Joët, Dominique Rumeau, Laurent Cournac, Gabor V. Horváth, Tony A. Kavanagh, Christian Schäfer[3], Gilles Peltier, and Peter Medgyesy*

Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H–6701 Szeged, Hungary (E.M.H., G.V.H., P.M.); Botanisches Institut, Universität Bayreuth, D–95440 Bayreuth, Germany (S.O.P., C.S.); Commissariat à l'Energie Atomique Cadarache, Département d'Ecophysiologie Végétale et Microbiologie, Laboratoire d'Ecophysiologie de la Photosynthèse, F–13108 Saint-Paul-lez-Durance, France (T.J., D.R., L.C., G.P.); and Department of Genetics, Trinity College, University of Dublin, Dublin 2, Ireland (T.A.K.)

The *ndh* genes encoding for the subunits of NAD(P)H dehydrogenase complex represent the largest family of plastid genes without a clearly defined function. Tobacco (*Nicotiana tabacum*) plastid transformants were produced in which the *ndhB* gene was inactivated by replacing it with a mutant version possessing translational stops in the coding region. Western-blot analysis indicated that no functional NAD(P)H dehydrogenase complex can be assembled in the plastid transformants. Chlorophyll fluorescence measurements showed that dark reduction of the plastoquinone pool by stromal reductants was impaired in *ndhB*-inactivated plants. Both the phenotype and photosynthetic performance of the plastid transformants was completely normal under favorable conditions. However, an enhanced growth retardation of *ndhB*-inactivated plants was revealed under humidity stress conditions causing a moderate decline in photosynthesis via stomatal closure. This distinctive phenotype was mimicked under normal humidity by spraying plants with abscisic acid. Measurements of CO_2 fixation demonstrated an enhanced decline in photosynthesis in the mutant plants under humidity stress, which could be restored to wild-type levels by elevating the external CO_2 concentration. These results suggest that the plastid NAD(P)H:plastoquinone oxidoreductase in tobacco performs a significant physiological role by facilitating photosynthesis at moderate CO_2 limitation.

Comparative analyses of the completely sequenced plastid genomes of such taxonomically distant plant species as liverwort (Ohyama et al., 1986), tobacco (*Nicotiana tabacum*; Shinozaki et al., 1986), and rice (Hiratsuka et al., 1989) has revealed a set of genes showing a surprising homology to subunits of the mitochondrial NADH dehydrogenase complex. This set of *ndh* genes proved to contain at least 11 members (Fearnley et al., 1989; Videira et al., 1990; Dupuis et al., 1991; Masui et al., 1991; Pilkington et al., 1991; Arizmendi et al., 1992), which are represented in all vascular plant divisions (Meng et al., 1986; Maier et al., 1995; Neyland and Urbatsch, 1996). The deduced amino acid sequence of the subunits of this plastid NAD(P)H dehydrogenase (NDH) complex shows significant homology with that of the corresponding subunits of the bacterial proton-pumping NADH: ubiquinone oxidoreductase and with the appropriate subunits of mammalian, fungal, and plant mitochondrial complex I (Fearnley and Walker, 1992; Weidner et al., 1993; Rasmusson et al., 1998). Subunits forming the highly conserved NADH-binding unit of complex I are apparently absent in the plastid NDH complex (Friedrich et al., 1995). However, this module might correspond to the additional, still uncharacterized (and presumably nuclear-encoded) subunits detected in the plastid NDH complex by biochemical methods (Quiles and Cuello, 1998; Sazanov et al., 1998b).

Plastid *ndh* genes are transcribed (Matsubayashi et al., 1987; Kanno and Hirai, 1993), the mRNAs are edited (Freyer et al., 1995; Maier et al., 1995), and the protein products of these genes are located in the stromal thylakoid membranes (Nixon et al., 1989; Berger et al., 1993; Kubicki et al., 1996). Expression of the various genes under different developmental and environmental conditions has been studied primarily in monocotyledonous plants (Kubicki et al., 1996; Martín et al., 1996; Catalá et al., 1997; Fischer et al., 1997). On the basis of western-blot analyses these investigators suggested that NDH proteins are primarily expressed in tissues of limited photosynthetic capacity. However, the expres-

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[2] Present address: Institute of Plant Sciences, Eidgenössische Technische Hochschule Center LFW C33, Universitätstrasse 2, CH–8092 Zurich, Switzerland.

[3] Present address: Molekulare Pflanzenphysiologie, Fachbereich 2: Biologie/Chemie, Universität Bremen, Postfach 33 04 40, D–28334 Bremen, Germany.

* Corresponding author; e-mail pmedgyesy@matavnet.hu; fax 36–62–433434.

sion of the *ndhD* gene in tobacco, measured as the extent of RNA editing creating the start codon, was restricted to chloroplasts and was highest in young, illuminated, photosynthetically active leaves (Hirose and Sugiura, 1997). Light activation of the thylakoidal NDH activity has also been recently demonstrated (Teicher and Scheller, 1998).

The longstanding question of the function of this putative NAD(P)H:plastoquinone (PQ) oxidoreductase in plastids has recently been tackled by targeted inactivation of several of the plastid-encoded *ndh* genes (*ndhA*, *B*, *C*, *H*, *I*, *J*, and *K*) in tobacco (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). A common feature of these NDH-inactivated plastid transformant plants is the absence of a transient increase in postillumination chlorophyll fluorescence, the presence of which is interpreted as a dark reduction of the PQ pool (Groom et al., 1993; Feild et al., 1998). On the basis of these results it was concluded that the NDH complex is functional in tobacco chloroplasts, mediating donation of electrons from a stromal reductant to the PQ pool in the dark (Burrows et al., 1998; Endo et al., 1998; Kofer et al., 1998; Sazanov et al., 1998a; Shikanai et al., 1998). These plastid transformant plants showed the normal characteristics of steady-state photosynthesis and, although water stress seemed to delay non-photochemical fluorescence quenching during induction of photosynthesis in certain mutants (Burrows et al., 1998), no NDH-specific phenotype was observed under normal or various stress conditions (Burrows et al., 1998; Sazanov et al., 1998a; Shikanai et al., 1998). In one laboratory the primary regenerants showed various abnormalities but neither linkage with the inactivated *ndh* genes nor cytoplasmic inheritance of these traits was demonstrated (Kofer et al., 1998). Therefore, the role of the NDH complex in the light reactions of photosynthesis and its physiological role in higher plants has remained hypothetical, and the various controversial conclusions are a matter of extensive discussion (Koop et al., 1998; Maliga and Nixon, 1998; Nixon and Maliga 1999; Roldán, 1999).

In the present study we have produced tobacco plastid transformants in which the *ndhB* gene was translationally inactivated. Preliminary data obtained on chlorophyll fluorescence transients under illumination showed marked differences between *ndhB*-inactivated and wild-type plants under anaerobic conditions (Cournac et al., 1998; Joët et al., 1998). Whereas this observation demonstrated the functioning of NDH complex during photosynthesis, since it was based on anaerobic conditions lacking both CO_2 and O_2, it was difficult to predict what natural physiological conditions might reveal a role for NDH. We show that under conditions that do not block but moderately inhibit photosynthesis by CO_2 limitation, the lack of NDH activity results in an enhanced growth retardation of *ndhB*-inactivated tobacco plants in comparison with the wild type.

RESULTS

Targeted Inactivation of the Plastid *ndhB* Gene in Tobacco

The *ndhB* is the only gene of the plastid *ndh* family located in the inverted repeat (IR_A and IR_B) region of the tobacco plastid genome (Shinozaki et al., 1986) and is most probably a part of the *rps12*(3′)-*rps7-ndhB-trnL* transcription unit (Matsubayashi et al., 1987; Kanno and Hirai, 1993). Inactivation of *ndhB* was accomplished by creating translational stop codons in the coding region of the gene. The pSSH1 plastid transformation plasmid contains a nightshade (*Solanum nigrum* L.) plastid DNA fragment and possesses mutations conferring spectinomycin and streptomycin insensitivity (Kavanagh et al., 1994, 1999). In the 7.8-kb inverted repeat region covered by the insert there is a 2.4% nucleotide sequence divergence between tobacco and nightshade plastid DNA (Kavanagh et al., 1999). The insert spans the first 732 nucleotides of *ndhB* (Wakasugi et al., 1998), which are identical in tobacco and nightshade. A single C was introduced into codon 206 of *ndhB* by oligonucleotide-directed mutagenesis of the plasmid in a region of the gene showing no editing site in tobacco (Freyer et al., 1995). This additional nucleotide generated a *Sma*I site and, in addition to a frame shift, stop codons (Fig. 1).

The mutant plasmid (pSSH1M) was introduced into tobacco protoplasts by polyethylene glycol treatment (O'Neill et al., 1993). Putative plastid transformant colonies were selected on the basis of their green color in a medium containing spectinomycin. The distinction between transformed and non-transformed tobacco plastids was facilitated, in addition to their insensitivity to streptomycin, by diagnostic RFLP differences in respect of the restriction enzymes *Sma*I, *Bam*HI, *Xho*I,

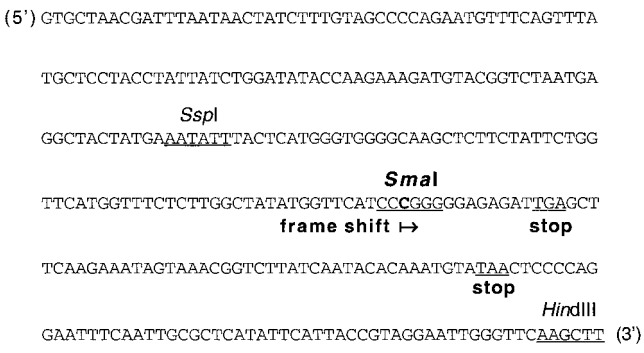

Figure 1. Translational inactivation of the *ndhB* gene in the pSSH1 plastid transformation plasmid. The pSSH1 plasmid insert spans the first 732 bp of the *ndhB* gene. An additional C-G bp was introduced into codon 206 of *ndhB* by oligonucleotide-directed mutagenesis. The resulting plasmid was called pSSH1M. This additional nucleotide generated a diagnostic *Sma*I site and, in addition to a frame shift, all three stop codons (only two of which are shown). A 300-bp portion of the *ndhB* coding region (identical in tobacco and nightshade) adjacent to the *Hind*III cloning site is shown as it appears in IR_A (5′–3′ direction, strand A), from position 143,798 in the tobacco plastid genome. Selected restriction enzyme sites are also shown.

and *Aat*II (Kavanagh et al., 1999). The selected lines comprised only 20% spontaneous spectinomycin-resistant mutants. On average one plastid transformant callus was selected in 10^4 viable colonies (or in 10^5 protoplasts treated). Each primary regenerant and its seed progeny was completely homoplasmic for the resistance markers. The polymorphic DNA regions (revealed as RFLPs between the donor and the recipient plastid DNA) were shown to be homoplasmic in all but one of the transformants demonstrating complete intraorganellar plastid DNA segregation after transformation (for representative *Sma*I patterns, see Fig. 3). A compilation of the RFLP and genetic markers in all of the 18 transformants revealed a high-frequency co-integration of the non-selected markers (Fig. 2). Nevertheless, the integration of the homeologous nightshade plastid DNA was mediated by multiple recombination events (Fig. 2). A schematic interpretation of the postulated recombination events following transformation of tobacco plastids with the pSSH1M plasmid in the individual transformants has been published elsewhere (Kavanagh et al., 1999). Because of the remarkably high recombination frequency in the 113-bp homologous peripheral region located between the *Sma*I site and the pUC19 vector (Fig. 2), one-half of the plastid transformants possessed the mutated (and putatively inactivated) *ndhB* gene.

Molecular and Biochemical Analysis of the Inactivation of the NDH Complex

Homoplasmy of selected transformants for the mutation inactivating the *ndhB* gene was also verified by Southern hybridization and PCR analysis. Probing of *Sma*I-digested plastid DNA by a plastid DNA probe covering the diagnostic *Sma*I site revealed both the site-specificity and homoplasmy of the introduced mutation (Fig. 3). This was further verified by *Sma*I digestion of PCR products generated using isolated plastid DNA as template and primers flanking the diagnostic *Sma*I site on the plastid genome (Fig. 4). In the subsequent investigations, unless stated otherwise, both wild-type tobacco and a double-resistant tobacco plastid transformant (possessing the full nightshade insert of the original pSSH1 plasmid) were used as controls. Furthermore, in most of the investigations two types of *ndhB*-inactivated transformant were used: number 1.2 was resistant to both streptomycin and spectinomycin, whereas number 3.3 contained only the spectinomycin-insensitivity mutation.

The fate of the NDH complex in the *ndhB*-inactivated plants was investigated by protein analysis. Fractioned chloroplast protein extracts were analyzed by PAGE and Coomassie Blue staining. No obvious difference was observed between the protein patterns of control (noninactivated transformant) and *ndhB*$^-$ plants (Fig. 5). As has been previously demonstrated (Burrows et al., 1998; Sazanov et al., 1998b) the NDH complex has a very low abundance in the thylakoid membrane. Attempts to produce a recombinant NDH-B polypeptide were not successful probably due to the high overall hydrophobicity of the NDH-B protein (Fearnley and Walker, 1992). Therefore, antibodies raised against NDH-H were used for western-blot analysis of chloroplast proteins. Immunostaining with the NDH-H antiserum confirmed the localization of NDH pro-

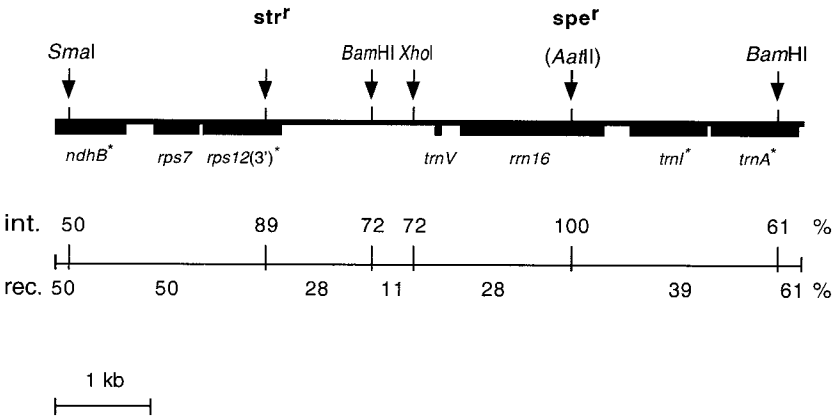

Figure 2. Distribution of co-integration and recombination frequencies in the targeted region following transformation of tobacco plastids with the pSSH1M plasmid. The 7.8-kb donor insert of the pSSH1M plasmid is shown at the top of the figure. Arrows mark the location of the specific resistance and RFLP sites scored (brackets indicate the absence of the wild-type restriction enzyme site). Asterisks mark intron-containing genes. At the lower part of the figure the line is sectioned to show the major intervals between the donor-type marker sites investigated in the plastid transformants. The frequency of co-integration (int.) of the individual non-selected markers with the selected spectinomycin resistance locus is shown above the line. A 100% value represents the total number of spectinomycin-resistant transformants possessing a donor marker. The observed recombination frequency (rec.) in the individual internal sections, calculated as a percentage of the transformants recombined in the particular interval, is shown below the line. A 100% value represents the total number of transformants possessing a recombination event.

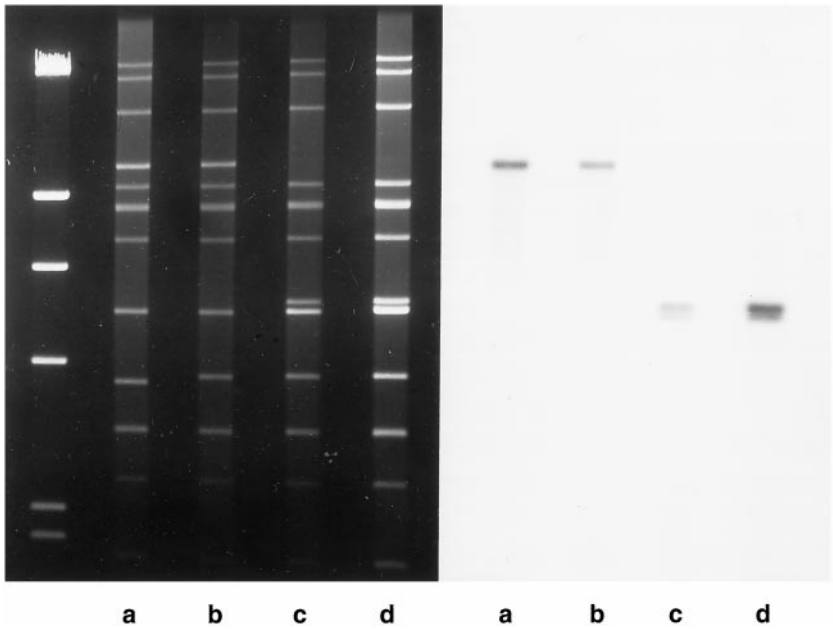

Figure 3. Site-specific inactivation of the *ndhB* gene in the plastid transformants. Gel electrophoresis of *Sma*I-digested plastid DNA of wild-type tobacco (a) and several plastid transformants (b–d) distinguishes a noninactivated transformant (b) from those possessing the inactivated *ndhB* gene (c–d). The smaller of the new, inactivation-specific fragments (5.68 and 5.45 kb) comigrates with the unchanged fragment number 9. On the left a *Hin*dIII digest of λ DNA is also shown (fragment sizes: 23.13, 9.42, 6.56, 4.36, 2.32, and 2.03 kb). Southern hybridization with a plastid DNA probe spanning the region containing the diagnostic restriction site in the 11.13-kb *Sma*I fragment number 4 reveals both the site-specificity and homoplasmy of the introduced mutation.

teins in the stroma lamellae of thylakoid membranes (Nixon et al., 1989; Berger et al., 1993; Kubicki et al., 1996) in control plants (Fig. 5). However, the western-blot analysis detected no NDH-H protein in the *ndhB*$^-$ plastid transformant investigated (Fig. 5), indicating that no functional NDH complex can be assembled. This conclusion is supported by similar observations in other tobacco plastid transformants where the inactivation of one of the *ndh* genes resulted in disappearance of the other NDH subunits investigated, even if they were encoded by separate transcription units (Burrows et al., 1998; Kofer et al., 1998).

Growth and Photosynthetic Performance of the NDH-Inactivated Transformants under Normal Conditions

Plastid transformants with a wild-type or an inactivated *ndhB* gene showed no visible NDH-specific phenotype under standard growth conditions either when cultured in vitro (autotrophically or on sugar-containing medium) or grown in soil in the greenhouse. Several of the primary regenerants showed morphological abnormalities (e.g. slow growth, distorted leaves, and poor pollen production) typical of chromosomal aneuploidy detected regularly in a certain percentage of tobacco plants regenerated from cell culture (Thanh et al., 1988). These morphological deviations did not show maternal inheritance and were not observed in the progeny obtained after pollination with wild-type tobacco. Transformant plants derived from three crosses with wild-type tobacco were analyzed in detail. No developmental deviation was observed in *ndhB*$^-$ plants compared with wild type from seed germination to seed set, including aging.

Measurements of photosynthetic CO_2 fixation or O_2 evolution in plants grown either in vitro or in soil showed no significant difference in steady-state photosynthetic rates between controls and *ndhB*$^-$ mutants. The in vivo light dependence curves of photosynthetic oxygen evolution were similar in control and *ndhB*$^-$ plants, indicating that neither the maximum efficiency nor the light-saturated capacity were affected in intact leaves under normal conditions (data not shown). Chlorophyll fluorescence measurements performed during dark-light-dark transitions indicated no difference in photochemical and non-photochemical quenching processes between control and *ndhB*$^-$ plants (Cournac et al., 1998). In these fluorescence measurements, following light extinction, a transitory and slow increase in the fluorescence level, before reaching the F_0 level, was observed in control plants. This phenomenon, generally ascribed to the dark reduction of the PQ pool by stromal reductants like NADPH or NADH (Groom et al., 1993), was not observed in *ndhB*$^-$ transformants (Cournac et al., 1998), indicating that stromal reductants in the dark do not reduce the PQ pool in the absence of the *ndhB* gene product. This impaired non-photochemical PQ reduction is apparently a standard feature of *ndh* gene-inactivated mutants (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998) and is the primary basis for the conclusion that the NDH complex is functional in chloroplasts (see the introduction). However the absence of any obvious phenotype in our *ndhB*$^-$ mutants, as was reported for other *ndhB*$^-$ mutants (Shikanai et al., 1998), supports the conclusion that under favorable growth conditions NDH function is dispensable. All these data prompted an extensive

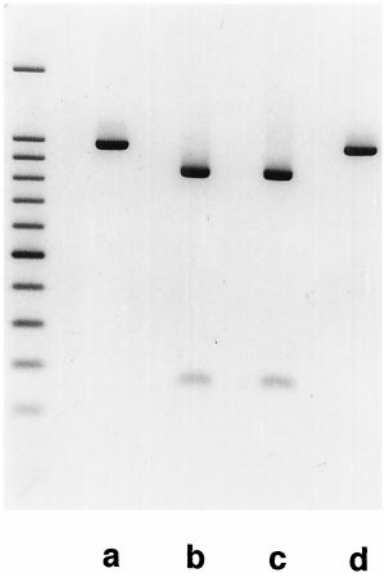

Figure 4. Homoplasmy of the plastid DNA population in the *ndhB*-inactivated transformants. Gel electrophoresis of *Sma*I-digested PCR product of wild-type tobacco (a), two *ndhB*-inactivated transformants (b and c), and a noninactivated transformant (d). The priming sites were located to cover the 5′ end of *ndhB* and flank the diagnostic *Sma*I site. The primer located inside the *ndhB* gene is outside the targeted plastid DNA region. The primers amplify a product of 966 bp, which is cut into 814- and 152-bp fragments by *Sma*I if the mutation introduced into *ndhB* is present. On the left a 100-bp DNA ladder is also shown (fragment sizes: 1,500 and 1,000–100 bp). The complete and correct cleavage of the PCR product in the *ndhB*-inactivated transformants reveals both the homoplasmy and site-specificity of the introduced mutation.

search for potential NDH function-specific stress conditions.

A Decrease in Air Humidity Generates a Discriminating Phenotype in NDH-Inactivated Transformants

We have investigated the effect of various stress conditions that have been proposed to stimulate cyclic electron transport activity (Heber and Walker, 1992; Fork and Herbert, 1993). The effect of a moderately long light stress (5 h, 700 μmol m^{-2} s^{-1}) on light dependence curves of photosynthetic O_2 evolution were measured in in vitro-grown *ndhB*-inactivated and noninactivated transformants. Neither the maximum efficiency nor the light-saturated capacity of photosynthetic O_2 evolution were differentially affected in intact leaves (data not shown). The effect of light stress on soil-grown plants was investigated in growth chambers. No significant difference was observed in photosynthesis performance (measured as whole-plant CO_2 fixation) of *ndhB*-inactivated and noninactivated transformants grown under an illumination of 1,200 μmol m^{-2} s^{-1} (16-h day) for 2 weeks (data not shown).

The effect of water stress (by severely limiting or withholding water) was also investigated on control and *ndhB*$^-$ plants grown under normal phytotron conditions. Under such stress conditions both types of plants showed a similarly strong (gradual or immediate) inhibition of vegetative development, in addition to a similar degree of wilting, yellowing, and withering of the older leaves. However, natural drought typically involves not only a soil water deficiency but also a dry atmosphere. Therefore, in another experiment we investigated the effect of low air humidity (30 relative %) on well-watered plants in a growth chamber. A few days of growth under these conditions surprisingly resulted in a remarkable growth difference between wild-type and *ndhB*$^-$ plants (for a representative pair of plants, see Fig. 6). After a 5-d-long growth period in low air humidity both fresh and dry weights of *ndhB*$^-$ plants (19.04 $\pm$ 2.05 and 1.38 $\pm$ 0.10 g, respectively) were almost 20% lower than those of wild-type plants (22.83 $\pm$ 1.16 and 1.70 $\pm$ 0.03 g, respectively). The enhanced growth retardation of *ndhB*$^-$ plants was visible primarily as a reduced growth of the young, expanding leaves (Fig. 6). No other phenotypic difference between the mutant and wild-type plants was detected in this experiment. The increased sensitivity to air humidity of mutants with a non-functional NDH

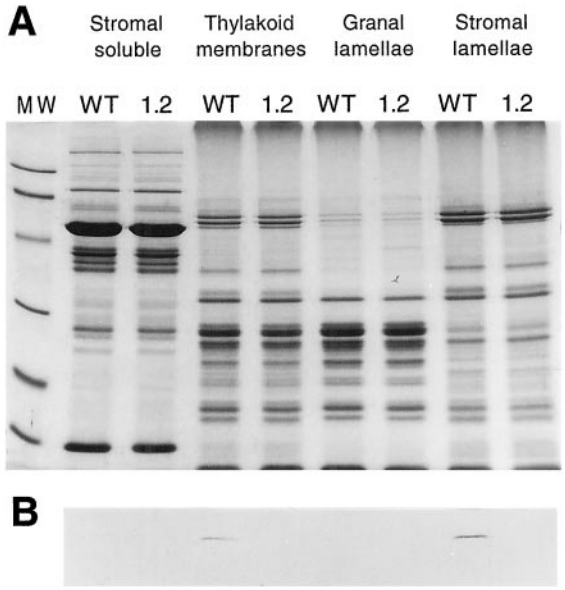

Figure 5. Absence of the NDH-H subunit in the *ndhB*$^-$ plastid transformants. Separation of protein fractions (20 mg of protein per lane) derived from purified chloroplasts by fully denaturating PAGE reveals no obvious difference after Coomassie Brilliant Blue staining between wild-type (WT) and *ndhB*$^-$ (1.2) tobacco plants. Western hybridization of the separated protein fractions electrotransferred onto nitrocellulose membranes by an anti-NDH-H antibody detects the protein in total thylakoid membranes and the stroma lamellae of the wild type. The absence of detectable NDH-H protein in the *ndhB*$^-$ mutant indicates that no functional NDH complex can be assembled. Molecular mass markers: 96, 66.2, 45, 31, 21.5, and 14.4 kD.

Figure 6. Enhanced growth delay of the *ndhB*$^{-}$ plastid transformants under humidity stress. Well-watered wild-type (left) and *ndhB*$^{-}$ (right) plants were grown in low air humidity (30% and 40% relative humidity during the day and the night, respectively), following a month of growth under normal conditions (60% relative humidity). A visible growth difference was developed in less than a week under humidity stress. The development of freshly expanding leaves was specifically hindered in *ndhB*$^{-}$ plants.

complex prompted an analysis of gas exchange under humidity stress.

Enhanced Reduction of Photosynthesis under Conditions Generating Moderate Stomatal Closure in NDH-Inactivated Transformants

Measurement of total CO_2 exchange by intact soil-grown plants was recorded continuously in computer-controlled growth chambers. The vigorous development of young wild-type tobacco plantlets in normal air humidity (60 relative %) was manifested as a steady day-by-day increase in the levels of whole-plant CO_2 fixation, whereas under conditions of low air humidity (30 relative %) photosynthetic development was considerably delayed (data not shown). Under normal growth conditions both wild-type and *ndhB*$^{-}$ plants displayed similar photosynthetic activity. In contrast, after a transition to low air humidity *ndhB*$^{-}$ plants showed a reduced photosynthesis that was up to 20% lower than that of wild-type plants (Fig. 7). These results indicated a primary role of humidity stress in triggering the cascade of events leading to a differential decline in photosynthesis and, concomitantly, growth. Therefore, the direct role of stomatal closure in generating the mutant-specific phenotype was investigated. Stomatal closure was induced in well-watered plants grown in normal air humidity by spraying with abscisic acid (ABA). A moderate treatment (spraying with a 10-μM ABA solution every 2nd d) resulted in a clearly visible growth difference between wild-type and mutant plants under normal phytotron conditions (Fig. 8). After a 2-week-long ABA treatment both fresh and dry

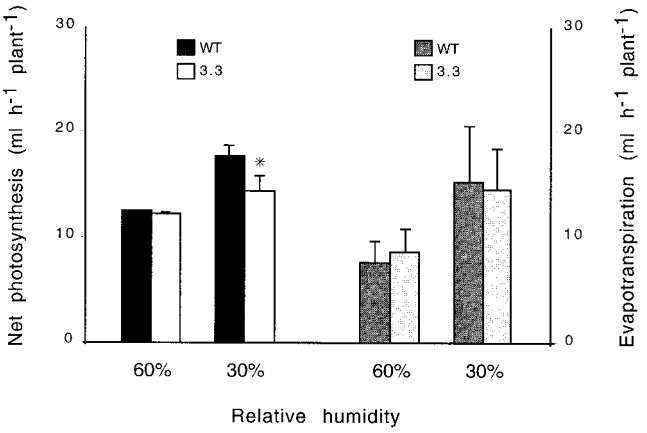

Figure 7. Differential reduction in photosynthesis of wild-type and NDH-inactivated plants grown in low air humidity. Photosynthesis and evapotranspiration of 5-week-old wild-type and *ndhB*$^{-}$ plants are shown in normal (60 relative %) and low (30 relative %) air humidity on the day preceding and following the humidity transition, respectively. The whole-plant photosynthesis and evapotranspiration values recorded in one experiment were the sum of four to eight plants of the same type grown in one computer-controlled growth chamber recording CO_2 consumption and water vapor condensation. On the left net photosynthesis calculated for one plant is displayed as the mean ± SD of four independent experiments. On the right evapotranspiration calculated for one plant is displayed as the mean ± SD of four independent experiments. Asterisk indicates significant differences from controls ($P < 0.05$). Low air humidity generated a difference in photosynthesis of *ndhB*$^{-}$ and wild-type plants, demonstrating an enhanced sensitivity of the *ndhB*$^{-}$ transformants to humidity stress. Evapotranspiration of *ndhB*$^{-}$ and wild-type plants showed a similar response, indicating that their stomata responded non-differentially to low air humidity.

Figure 8. ABA treatment provokes the mutant-specific stress phenotype under normal growth conditions. Wild-type (left) and *ndhB*$^{-}$ (right) plants grown for a month under normal phytotron conditions were subsequently sprayed with 10 μM ABA solution. The growth difference was developed during 2 weeks of spraying of the leaves every 2nd d. The development of freshly expanding leaves was specifically hindered in *ndhB*$^{-}$ plants.

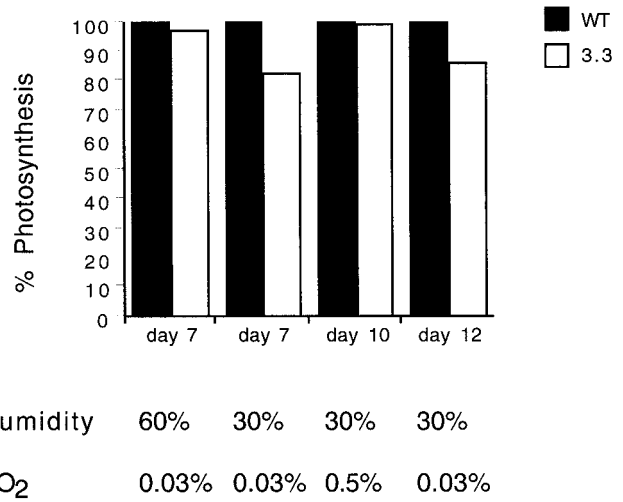

Figure 9. Elevated external CO_2 concentration complements the differential photosynthesis reduction generated by humidity stress. Five-week-old wild-type and *ndhB*$^-$ plantlets were grown under normal conditions in computer-controlled growth chambers recording CO_2 exchange. The effect of humidity stress (caused by decreasing the relative air humidity from 60% to 30%) and the additional effect of elevated CO_2 concentration (from ambient to 0.5%) was tested. Whole-plant photosynthesis in one chamber during illumination was recorded as the CO_2 consumption of six plants of the same type. Relative photosynthesis values are displayed on the *y* axis. For ease of comparison, photosynthesis of wild-type plants was taken to be 100%. The mean wild-type absolute photosynthesis values (from left to right) were the following: 15.55, 18.06, 54.26, and 31.51 mL^{-1} h^{-1} $plant^{-1}$. The relative decrease in whole-plant photosynthesis in NDH-inactivated plants was fully compensated during the transitory elevation of the CO_2 level. This result pinpoints limitation of CO_2 availability as a direct cause of the differential reduction in photosynthesis in wild-type and *ndhB*$^-$ transformant plants under humidity stress conditions.

weights of *ndhB*$^-$ plants (18.40 ± 1.20 and 0.93 ± 0.11 g, respectively) were almost 25% lower than those of wild-type plants (23.84 ± 3.60 and 1.25 ± 0.27 g, respectively). Similar to the effect of humidity stress, the differential phenotype appeared primarily as a reduced growth of the young, expanding leaves of *ndhB*$^-$ plants (Fig. 8). It was notable that a strong ABA treatment (daily spraying with 20 μM ABA) resulted in a strong but non-differential growth inhibition of both wild-type and mutant plants, similar to that caused by water stress (data not shown). These results suggested that a moderate increase in stomatal resistance is an important intermediary in the process leading to a decline in photosynthesis in low air humidity. Therefore, we measured stomatal conductance changes occurring during the transition from high (75 relative %) to low (30 relative %) air humidity on the basis of gas exchange measurements on attached leaves. These investigations demonstrated a moderate and similar decline in leaf conductance in *ndhB*$^-$ mutants (from 22.73 ± 7.29 to 16.05 ± 6.13 mmol m^{-2} s^{-1}) and the wild type (from 23.61 ± 7.30 to 17.08 ± 5.58 mmol m^{-2} s^{-1}). In line with these results whole-plant evapotranspiration rates of *ndhB*$^-$ and wild-type plants measured in growth chambers showed a similar increase after a transition to low air humidity (Fig. 7). We have concluded from these observations that reduced CO_2 availability, due to the (non-differential) stomatal response to low air humidity, is the principle factor generating the mutant-specific phenotype.

Our conclusion that a decline in internal CO_2 concentration is the key element in the cascade of events leading to the differential inhibition of photosynthesis was further investigated by photosynthesis measurements on plants simultaneously grown in low air humidity and elevated external CO_2 concentration. Low air humidity triggered a greater decrease in the photosynthetic capacity of *ndhB*$^-$ plants in comparison with that of wild-type plants. However, increasing the ambient CO_2 level to 0.5% resulted in the disappearance of the difference in photosynthesis levels while markedly increasing overall levels in both types of plant (Fig. 9). When the ambient CO_2 concentration was returned to normal, the differential effect of low air humidity on photosynthesis levels re-appeared. The above data strongly support the view that the differential effect of low air humidity on NDH-inactivated plants is implemented by a differential sensitivity of photosynthesis to limiting CO_2 availability. Our observations also demonstrate that the lack of a functional NDH complex is primarily manifested at a level of CO_2 limitation that does not strongly inhibit the growth and photosynthetic development of wild-type plants.

DISCUSSION

The low abundance of the chloroplast NDH complex (Burrows et al., 1998; Sazanov et al., 1998b) has hampered investigations into its molecular and physiological role. However, the recent application of plastid transformation techniques, which permit targeted inactivation of individual *ndh* genes, has greatly facilitated these investigations (see the introduction). In these experiments insertional mutagenesis or deletion of the gene was achieved via site-specific integration of a dominant selectable marker gene (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). Our experiments demonstrate that the *ndhB* gene can also be efficiently inactivated using a different strategy: translational inactivation by replacement of the wild-type plastid *ndhB* gene with a frame-shifted mutant produced by site-directed mutagenesis. In this approach a single nucleotide change was introduced into a cloned copy of the *ndhB* gene, which was located on the same DNA fragment several kilobase pair distant from a binding-type antibiotic insensitivity mutation in the *rrn16* gene. The latter gene was then used to select for plastid transformants in which both mutant genes had replaced their wild-type counterparts on the plastid genome. In our experiments the mutated *ndhB* gene was located close to the end of the trans-

forming DNA (0.1 kb from the junction with vector DNA) and at a distance of 5 kb from the spectinomycin insensitivity mutation used for selection purposes. Nevertheless, in 50% of the transformants the unselected mutation was co-integrated with the selectable marker and yielded a homozygous population of *ndhB*-inactivated plastid DNA, indicating high local recombination frequencies near the vector-insert junction. In homeologous plastid transformation experiments in *Nicotiana* sp. this phenomenon routinely results in recombination/integration frequencies up to 10 times higher than expected at the ends of the plastid DNA insert (Kavanagh et al., 1999). An additional remarkable observation was that our experiments revealed transformation efficiencies typical of those found in homologous plastid DNA transformations (Golds et al., 1993; O'Neill et al., 1993), despite the 2.4% nucleotide sequence divergence between tobacco and nightshade plastid DNA in the transformed region. This observation indicates the prevalence of a RecA-type homeologous recombination mechanism in higher plant plastids (for discussion, see Kavanagh et al., 1999) and suggests that plastid transformation vectors directed to this region do not need to be species specific, at least for species that show a similarly low degree of nucleotide sequence divergence.

The absence of any obvious specific phenotype in the *ndhB*-inactivated transformants when grown under favorable conditions supports earlier conclusions concerning the dispensability of NDH function (Burrows et al., 1998; Shikanai et al., 1998). In other experiments various abnormalities of *ndh*-inactivated primary regenerants were detected (Kofer et al., 1998), but since their linkage with the inactivated gene was not demonstrated, tissue culture effects cannot be excluded as a plausible explanation of the morphological deviations (Maliga and Nixon, 1998). We have shown that under conditions where air humidity is decreased or when plants are sprayed with ABA, photosynthesis is more significantly reduced in *ndhB*-inactivated transformants than in wild-type plants, and this effect on photosynthesis causes a corresponding reduction in biomass. This phenotypic difference was suppressed when ambient CO_2 concentration was increased, thus showing that it is likely mediated by stomatal closure triggered either by low air humidity or by ABA treatment (Downton et al., 1988; Robinson et al., 1988; Willmer and Fricker, 1996). Since no differences in transpiration rates were observed in either whole plants or leaves of controls and *ndhB*$^-$ mutants in response to changes in air humidity, we conclude that stomatal regulation was not affected in the mutant plants. Therefore, low internal CO_2 concentration resulting from partial stomatal closure is likely responsible for the observed phenotypic difference. In previous investigations *ndh*$^-$ mutants have been reported to display a reduced non-photochemical quenching of fluorescence during photosynthesis induction under water stress conditions (Burrows et al., 1998). However, no distinctive visible phenotype was observed by the authors in response to water stress (Burrows et al., 1998). We also observed that in conditions where stomatal closure is pronounced, which occurred in response either to a severe limitation in water supply or to spraying with a high ABA concentration, there was no phenotypic difference. This can be explained by the fact that under such conditions net photosynthesis and growth are so strongly inhibited in both types of plants that the presence or absence of a functional NDH complex has no discernible effect. This is in line with the observations that water stress decreases photosynthetic assimilation of CO_2 by metabolic inhibition (Tezara et al., 1999) and that fully functional photosynthesis is required for the humidity dependence of CO_2 assimilation to be manifested (Stitt et al., 1991). We conclude from our experiments that moderate inhibition of photosynthesis by CO_2 limitation can trigger a phenotypic difference between wild-type and *ndh*-inactivated plants.

Several processes are part of the photosynthetic controls that coordinate the synthesis of ATP and NADPH with their rate of use in carbon metabolism (Foyer et al., 1990; Heber and Walker, 1992). Metabolic demands can often require that light-dependent ATP production be increased relative to NADP reduction. Inhibition of the linear electron flow can occur if there is an imbalance between the stoichiometry of ATP/NADPH production and consumption. A common feature of the NDH-inactivated tobacco plants is the disappearance of a dark transient increase in fluorescence after illumination (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). This phenomenon has been ascribed to a dark reduction of the PQ pool by stromal reductants (Groom et al., 1993) and has been considered to be an after-effect of a light-dependent process, i.e. cyclic electron transport around photosystem I (Burrows et al., 1998; Shikanai et al., 1998). This auxiliary electron flow may modulate the ATP to NAD(P)H ratio by participating in the redox control of the PQ pool. However, different pathways of cyclic electron flow around photosystem I have been suggested to occur in chloroplasts, which are considered likely to involve the NDH complex or a ferredoxin:PQ oxidoreductase activity (Ravenel et al., 1994; Endo et al., 1997). Also, the recovery of the postillumination fluorescence increase in NDH-inactivated plants under certain stress or developmental conditions (Sazanov et al., 1998a; Shikanai et al., 1998) supports the existence of alternative pathways involved in non-photochemical PQ reduction. The absence of a phenotypic difference between wild-type and *ndhB*-inactivated plants when grown under normal conditions suggests that the alternative mechanisms can generate sufficient extra ATP. In contrast, under conditions where CO_2 availability decreases due to moderate stomatal closure, a differ-

ential phenotype is observed. Under such conditions photorespiratory activity is increased due to competition between CO_2 and O_2 at the Rubisco catalytic site. It has been reported that the requirement for ATP is increased during photorespiration (Osmond, 1981). Therefore, we propose that the alternative pathways involved in the production of extra ATP are not efficient enough to fulfill the higher ATP demand of active photosynthesis when photorespiration is operating at a high rate. This would explain why the phenotypic differences between wild-type and NDH-inactivated plants are observed only under conditions that result in moderate CO_2 limitation.

Field-grown plants typically experience extensive periods during which the evaporative demand exceeds the water supply. These conditions can occur in the absence of severe soil water deficiency, e.g. because of fluctuating water inputs from rainfall or irrigation in a dry atmosphere. Under such conditions, the sensitive response of stomata to humidity as the environmental evaporative demand changes provides an efficient means by which tissue water deficits can be avoided. As a consequence, photosynthetic tissues will be subjected to partial stomatal closure for extensive time periods. Under these conditions, in which photosynthesis is limited by CO_2 availability, extra ATP production through an NAD(P)H:PQ oxidoreductase-dependent pathway may confer a selective advantage of sufficient magnitude to explain the conservation of plastid *ndh* genes during the course of evolution.

MATERIALS AND METHODS

Plasmid Construction

The pSSH1 plasmid (Kavanagh et al., 1999) contains a 7.8-kb *Hin*dIII fragment cloned from a black nightshade (*Solanum nigrum*) plastid double mutant (McCabe et al., 1989; Kavanagh et al., 1994). The pSSH1 mutations confer spectinomycin and streptomycin insensitivity and are located in the *rrn16* and the *rps12*(3′) genes, respectively. A 732-bp initial portion of the *ndhB* gene is located at one end of the cloned cpDNA insert. The nucleotide sequence of this region of *ndhB* is identical in both tobacco (*Nicotiana tabacum*) (Shinozaki et al., 1986; GenBank accession no. Z00044) and nightshade (Kavanagh et al., 1999; EMBL accession no. Y18934). Oligonucleotide-directed mutagenesis of the *ndhB* gene was performed using the in vitro mutagenesis system (Altered Sites II, Promega, Madison, WI). The *Hin*dIII fragment from pSSH1 was cloned into the pAlter-1 vector and was mutagenized using the following mutagenic oligonucleotide: 5′-AATCTCTCCCCCGGGATGAACCATA-3′.

Plastid Transformation

Tobacco (*N. tabacum* L. cv Petit Havana) was maintained as shoot cultures on agar-solidified Murashige and Skoog medium (Murashige and Skoog, 1962) in the light (50 μmol m^{-2} s^{-1}, 16-h day, 25°C). Polyethylene glycol-mediated plastid transformation was performed as described (O'Neill et al., 1993). The selective medium contained 1,000 mg L^{-1} spectinomycin dihydrochloride. Plants were regenerated from the resistant colonies, and leaf calli and seedlings were tested for their resistance as described (Cséplő, 1994; Medgyesy, 1994). In the resistance tests spectinomycin dihydrochloride and streptomycin sulfate were used separately at 1,000 mg L^{-1} each.

Plastid DNA Analysis

Chloroplasts were isolated from aseptically grown plants according to Bookjans et al. (1984). Lysis of chloroplasts, the purification of DNA, and the RFLP analysis followed standard protocols (Sambrook et al., 1989). Nonradioactive Southern hybridization was performed using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). *Sma*I-digested plastid DNA separated by horizontal agarose slab-gel electrophoresis and visualized by ethidium bromide staining was denatured and bound to positively charged nylon membranes (Hybond N+, Amersham, Arlington Heights, IL) according to standard protocols. The probe was a 483-bp *Nco*I-*Bsr*GI fragment of tobacco plastid DNA, which covered the diagnostic *Sma*I site. The nucleotide sequence of oligonucleotide primers that were used for PCR analysis of plastid transformants together with their position in IR_B within the tobacco plastid genome is as follows: 5′-ACGTCAGGAGTCCATTGATGA-3′ (98,495–98,515) and 5′-CGAAACAAACGAAAAGGAAAG-3′ (99,459–99,439). Efficient amplification was achieved using approximately 20 ng of plastid DNA in a 20-μL reaction using the PCR System of Fermentas (Vilnius, Lithuania) and the following cycle parameters: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; 25 cycles.

Preparation of Antibody against NDH-H

The tobacco *ndhH* gene that extends from nucleotide 123,672 to 124,910 (Shinozaki et al., 1986; GenBank accession no. Z00044) was PCR-amplified using *Pfu* polymerase (Stratagene, La Jolla, CA) and cloned by blunt-end ligation into the *Sma*I site of pGEX4-T3 (Pharmacia Biotech, Uppsala). The recombinant plasmid was transformed into the *Escherichia coli* strain DH5α (Gibco-BRL, Cergy Pontoise, France). The resulting clones were sequenced to ensure in-frame fusion of *ndhH* with the glutathione-*S*-transferase gene and to avoid clones that contained PCR-generated mutations. Overexpression of the GST−NDH-H fusion protein was induced with 50 μM isopropylthio-β-galactoside for 5 h at 37°C. Bacterial cultures were pelleted by centrifugation (2,000*g*, 10 min) and resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM Na_2-EDTA, and 100 mM NaCl. Fusion protein was extracted from inclusion bodies by standard procedure (Sambrook et al., 1989) and separated by SDS-PAGE. The relevant band was excised from the gel and the protein was electroeluted. Antiserum was raised against the fusion protein in rabbit (Bioenvirotech, Marseille, France).

Preparation of Thylakoid Membranes

Intact chloroplasts were isolated and purified from leaves using discontinuous Percoll (Pharmacia Biotech) gradients as described (Rumeau et al., 1996). Chloroplasts were osmotically lysed in MNM solution containing 20 mM MES (2-[*N*-morpholino]-ethanesulfonic acid), pH 6.0, 15 mM NaCl, and 5 mM $MgCl_2$, and centrifuged for 20 min at 35,000*g*. The supernatant fraction comprised stromal soluble proteins. Stromal and grana lamellae were separated following a stacking step carried out as described by Sazanov et al. (1998b). Briefly, thylakoid membranes were allowed to stack for 1 h and then solubilized by adding *n*-dodecyl-β-D-maltoside dropwise to 1% (w/v) with constant stirring. After incubation (30 min) insoluble material was removed by centrifugation at 1,000*g* for 2 min, and the different fractions were recovered by differential centrifugation. Grana thylakoids were recovered by centrifugation at 10,000*g* for 30 min and stroma thylakoids by centrifugation at 150,000*g* for 1 h.

PAGE and Immunodetection

Denaturing SDS-PAGE was performed as described by Laemmli (1970) using 13% (w/v) acrylamide gels. Proteins were either stained with Coomassie Brilliant Blue or electrotransferred onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and probed with NDH-H antibodies. Immunocomplexes were detected using alkaline phosphatase-conjugated antibodies.

O_2 Evolution Measurements in Plants Cultured in Vitro

Seedling-derived plants were grown in vitro as described (Peter et al., 1999). The leaf discs were collected from plantlets grown under 60 μmol m^{-2} s^{-1} photon flux density (PFD), illuminated by cool-white fluorescence tubes. The effect of light stress was analyzed by exposing the culture vessels to a PFD of approximately 700 μmol m^{-2} s^{-1}. The light was provided by low-voltage (12-V/50-W) multimirror halogen lamps (Precise, General Electric, Fairfield, CT). Three heat filters (Tempax, Schott, Cologne, Germany) and a water filter (10-cm height, 13°C) were placed between the culture vessel and the light source to minimize any temperature increase in the vessel. Light dependence curves of net oxygen evolution were measured at 25°C, approximately 2% (v/v) CO_2 (carbonate/bicarbonate buffer), with a leaf disc oxygen electrode (LD2, Hansatech, King's Lynn, UK) and a pulse-amplitude-modulation fluorometer (PAM, Walz, Effeltrich, Germany). The leaf discs were exposed to alternating light periods (7.5 min) and dark periods (5 min), and the PFD was raised at each light period up to 400 μmol m^{-2} s^{-2}. Gross photosynthesis was calculated from the difference of O_2 evolution rate in the light and in the consecutive dark period.

Leaf Conductance Measurements in Soil-Grown Plants

CO_2 and H_2O exchange of attached leaves of soil-grown plants under humidity transition was measured in an air-tight chamber. Relative humidity (75% and 30%) was set by generating moist air using a portable dew point generator (LI-610, LI-COR, Lincoln, NE) at a flow rate of 2 mL s^{-1}. The moist air was drawn into a leaf clip (PLC model, Ppsystem, Hotchin, UK), equipped with leaf ventillation, thermistor air temperature measurement, and infrared sensor leaf temperature measurement. A gas mixer (SEMY Engineering, Montpellier, France) was used to generate gas mixture with a defined CO_2 and O_2 concentration. CO_2 and O_2 concentrations were measured using an infrared gas analyzer (LI-6262, LI-COR, Lincoln, NE) and an oxygen analyzer (OXOR 6N, Maihak, Hamburg, Germany). CO_2 and H_2O exchange was measured by monitoring air humidity and CO_2 concentration changes in air between the inlet and outlet of the chamber. Standard calculations were used to determine stomatal conductance (Farquhar and Sharkey, 1982).

Whole-Plant Photosynthesis and Growth Measurements

Three-week-old in vitro-grown seedlings were potted into soil and grown for an additional 2 weeks in a phytotron or a growth chamber before using a stress condition. The standard conditions in the phytotron were 16-h light (250–350 μmol m^{-2} s^{-1} PFD, Osram HQI-T/DV lamps, 30°C–32°C), 8-h dark (22°C), 50% to 60% relative humidity. The plants were supplied six times a day by nutrient solution (one-half-diluted Hoagland salts; Hoagland and Arnon, 1950) in an excess amount resulting in over-dipping from the soil. ABA treatment was performed by spraying 15 to 25 μL of ABA solution (10 μM) on the lower surface of all leaves of a plant every 2nd d. The computer-controlled $C_2$3A system (Fabreguettes et al., 1994) consists of air-tight twin growth chambers suitable for comparative investigation of two sets of plants. The continuously adjusted and recorded parameters in the chambers (the standard values are indicated in brackets) were the following: CO_2 concentration (0.034%), O_2 concentration (16%), air humidity (60 relative %), evapotranspiration, temperature (day/night: 30°C/25°C). Illumination (16-h day) was provided by metal halogen lamps (Powerstar HQI-T/D, Osram, Munich), offering 250 and 350 μmol m^{-2} s^{-1} PFD at the level of upper leaves of young and mature (non-flowering) plants, respectively. Water was supplied normally four times a day as nutrient solution (one-half-diluted Hoagland salts; Hoagland and Arnon, 1950) in controlled amounts 20% to 50% more than the daily water loss by evapotranspiration. In the case of extended water stress by limited watering, after some days pure water was used to avoid salt accumulation. Humidity stress was achieved by specifying 30% and 40% relative humidity during day and night, respectively. The measurements of net photosynthesis and dark respiration were based on the quantitative balance of CO_2 injection and trapping, respectively, maintaining a constant CO_2 concentration (measured by an infrared gas analyzer) in the chambers (Fabreguettes et al., 1994). Air humidity was measured using, in addition to the in-built humidity detector, a portable humidity detector (Hydrodig 2010, Tecnic Instruments, Marseilles, France) at the level of leaves. Evapotranspiration was measured by

weighing the collected condensed water vapor. Six plants normally were grown in each chamber. The use of large growth chambers for whole-plant photosynthesis measurements does not easily allow statistical evaluation of individual plants, therefore the experiments were repeated to test their reproducibility.

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B. Non-photochemical reduction of intersystem electron carriers in chloroplasts of higher plants and algae

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NON-PHOTOCHEMICAL REDUCTION OF INTERSYSTEM ELECTRON CARRIERS IN CHLOROPLASTS OF HIGHER PLANTS AND ALGAE

[1]Laurent Cournac, [1]Geneviève Guedeney, [1]Thierry Joët, [1]Dominique Rumeau, [2]Gwendal Latouche, [2]Zoran Cerovic, [3]Kevin Redding, [4]Eva Horvath, [4]Peter Medgyesy, [1]Gilles Peltier

[1]CEA Cadarache, DEVM Bat 161, 13108 Saint-Paul-lez-Durance, France
[2]CNRS-LURE Bat 203, Centre Universitaire Paris-Sud, 91405 Orsay, France
[3]University of Geneva, 30, quai Ernst-Ansermet, CH-1211, Switzerland
[4]BRC, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

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1. Introduction

Besides reactions involved in the « Z » scheme of photosynthesis, additional electron transport pathways, such as cyclic electron flow around PS1 or chlororespiration, have been reported in algal and in higher plant chloroplasts (1-4). These pathways generally involve a reduction of the intersystem electron transport chain (plastoquinones and/or the cyt b_6f complex) by stromal components such as NAD(P)H or reduced ferredoxin. Different electron carriers have been proposed to mediate the non-photochemical reduction of inter-system electron transport chain. The discovery of chloroplast genes showing homologies with genes encoding subunits of the mitochondrial complex I (*ndh* genes), was taken as an evidence for the existence of a NADH dehydrogenase complex I in chloroplasts. Since, the existence of such a complex has been reported from non-denaturing gel electrophoresis (5) and recently, the NDH complex has been purified and partially characterized from pea chloroplasts (6). Inactivation of chloroplast *ndh* genes by plastid transformation has been undertaken in order to assess the role of the NDH complex during photosynthesis. However, if the disruption of *ndh* genes allowed to show that the NDH complex is functional *in vivo* and is involved in the dark re-reduction of the PQ pool after a period of illumination (7), a participation of the NDH complex during photosynthesis was not shown. Surprisingly, no clear phenotype was observed in inactivated transformants (7), suggesting that the NDH complex has a marginal role during photosynthesis. Other mediators have been proposed to be involved in the non-photochemical reduction of inter-system electron carriers : these include the putative ferredoxin-quinone-oxidoreductase activity (FQR), FNR (4) and more recently a NAD(P)H-PQ oxidoreductase activity characterized in potato chloroplasts that would be different from the NDH complex, from FNR, and from FQR (8).

Interestingly, although chlororespiration and cyclic electron flow around PS1 have been extensively studied in the green alga *Chlamydomonas*, sequencing of the chloroplast genome of different *Chlamydomonas* species has revealed the absence of *ndh* genes (9). One possibility would be that *ndh* genes have been transferred to the nuclear genome during the time course of evolution, but the high hydrophobicity of some *ndh* gene products makes this possibility rather unrealistic. Another possibility would be that in green algae like *Chlamydomonas*, a different mechanism is involved in the non-photochemical reduction of the PQ pool.

In the present paper, we have studied the nature of the electron carriers(s) involved in the non-photochemical reduction of the intersystem electron transport chain in higher plant chloroplasts and in *Chlamydomonas.* Targeted inactivation of the *ndh*B gene has been achieved by plastid transformation of tobacco protoplasts and the ability of exogenous NAD(P)H to donate electrons to the intersystem electron transport chain was studied in transformed plants. In *Chlamydomonas* cells, interaction between stromal components and the PQ pool was studied by measuring H_2 production in WT cells and O_2 exchange in PS1-deficient mutants. Results are discussed in relation to the existence of different pathways of PQ reduction in chloroplasts.

2. Material and Methods

2.1 Chlorophyll fluorescence measurements - chlorophyll fluorescence was measured on tobacco chloroplasts at 25°C using a pulse modulated amplitude fluorimeter (PAM 101-103, Walz, Effeltrich, Germany) as described in (8). Anaerobiosis was achieved by addition of glucose (20 mM) and glucose oxidase (2 $mg.ml^{-1}$) to the chloroplast suspension about 15 min before measurements. Blue green fluorescence measurements were made on a new version of the pulse modulated fluorometer described in (10).

2.2 Mass spectrometric measurement of gas exchange - algal cultures were harvested and 1.5 ml of the suspension was placed in the measuring chamber of the mass spectrometer. The experimental procedure for O_2 exchange assays has been described in (11). For measurements of H_2 evolution, cultures were subjected to anaerobiosis by sparging the sample with N_2, closing the chamber and leaving algae consume residual O_2 from the medium. When O_2 concentration was undetectable (< 0.2 µM), cultures were incubated for 30 min before starting measurements.

2.3 Plant material - *Chlamydomonas* strains deleted in *psaA* and *psa*B genes (*psa*AΔ and *psa*BΔ) were made as described earlier (12). The *Chlamydomonas* MUD2 mutant resistant to myxothiazol was generously supplied by Dr. P. Bennoun. Double mutants were produced by sexual crosses. Targeted inactivation of the *ndh*B gene was achieved in tobacco (Petit Havana) by replacing the gene with a mutant version possessing a translational stop in the coding region. The mutated gene was physically linked to selectable markers (spectinomycin and streptomycin resistance) located on the same plastid DNA fragment derived from a *Solanum nigrum* plastid mutant. Plastid transformants were produced via polyethylene glycol-mediated transformation of tobacco protoplasts, and selection for the binding-type antibiotic resistances was used to generate single-cell derived homoplasmic primary regenerates of plastid transformants (13). Physical mapping and Southern hybridation were performed to

confirm homoplasmy and the presence of the mutation in transformants. Control lines were generated by inserting the selectable markers alone.

3. Results

When *Chlamydomonas* cells are placed under anaerobic conditions, the chloroplast enzyme hydrogenase is induced and produces H_2 by using reduced ferredoxin as an electron donor. This reaction can function in the absence of PS2 activity (for instance in DCMU-treated algae), electrons being supplied to PS1 by the intersystem electron transport chain. This supplies a convenient experimental model to study *in vivo* reactions involved in the non-photochemical reduction of PQ. In normal conditions, H_2 production is transitory, being slowed by the generation of a thylakoid proton gradient and followed by activation of Calvin cycle CO_2 fixation. In the presence of uncouplers (2µM FCCP), H_2 production can be sustained, as proton gradient vanishes and CO_2 fixation, which needs ATP, is prevented. In the absence of DCMU, H_2 was produced at PS1 and O_2 was produced by PS2 (Fig. 1A). In the presence of DCMU, O_2 evolution was stopped and H_2 was still produced at a consequent rate (about 500 nmoles. $min^{-1}.mg^{-1}$chlorophyll). H_2 production measured in the presence of DCMU was insensitive to rotenone or to low concentrations of piericidin A, well-known inhibitors of complex I, thereby indicating that a complex I-like enzyme was likely not involved in this reaction. On the other hand, we found that H_2 production in the presence of DCMU was inhibited by N-ethylmaleimide (NEM - Fig. 1D) a sulfhydryl group reagent, although this compound did not affect PS2-driven H_2 production (Fig. 1C).

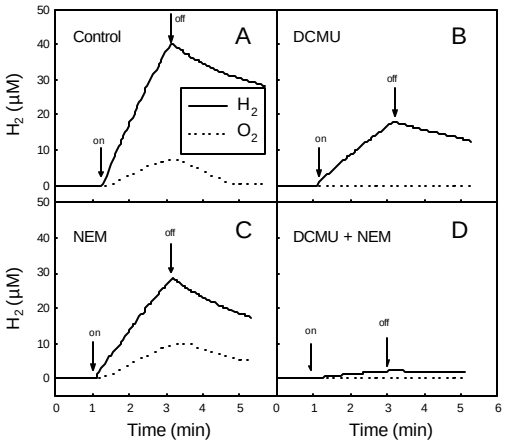

Fig 1 : Light-driven H_2 production by *Chlamydomonas* cells under anaerobic conditions. FCCP (2µM) was added before measurements. A : control ; B : in the presence of 10 µM DCMU ; C : in the presence of 1 mM NEM ; D : in the presence of 10 µM DCMU and 1 mM NEM.

In order to better characterize reactions involved in the reduction/oxidation of the PQ pool, we investigated O_2 exchange in *Chlamydomonas* mutants deleted in PS1. In such mutants, CO_2 fixation does not occur, but a PS2-dependent electron flow has been characterized by mass spectrometry (11), electrons produced at

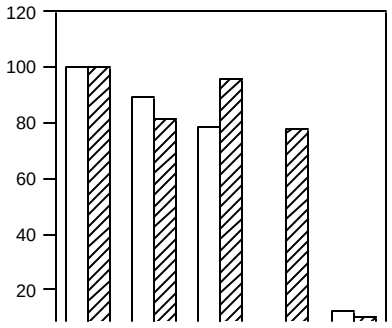

Fig 2 : Effect of mitochondrial inhibitors (SHAM : 0.4 mM; Mx : 2 µM myxothiazol; AntA : 2 µM antimycin A) on PS2-driven O_2 exchange in *Chlamydomonas* mutants *psa*BΔ (□) and *psa*BΔ MUD2 ( ).

PS2 being used for O_2 reduction. We found that the PS2-dependent electron flow was inhibited by DCMU, thus indicating the involvement of the PQ pool, and was unaffected in a mutant lacking the cyt b_6/f complex (not shown). Quite interestingly, the PS2-dependent pathway was sensitive to the presence of inhibitors of mitochondrial respiration (Fig. 2). Simultaneous addition of myxothiazol (an inhibitor of the mitochondrial cytochrome bc_1 complex) and of salicyl hydroxamic acid (SHAM - an inhibitor of the alternative oxidase) inhibited the PS2-dependent electron flow, whereas myxothiazol or SHAM alone did not, as is classicaly observed for inhibition of mitochondrial respiration. The inhibition was not observed in a mutant resistant to myxothiazol (MUD2), unless myxothiazol was replaced by an other inhibitor of the cytochrome oxidase pathway such as antimycin A. This clearly shows that this PS2-dependent flow needs ongoing mitochondrial respiration.

In order to investigate the nature of the interaction between chloroplasts and mitochondria, we monitored redox state variations of the NAD(P)H pool by measuring light-induced changes in blue-green fluorescence. In PS1 deficient mutants, the light-induced reduction of NAD(P)H is very small when compared to what occurs in WT (Fig. 3). When mitochondrial activity was blocked by addition of myxothiazol and SHAM, both the dark level of NAD(P)H reduction and the light-dependent increase were enhanced. The inhibition of PS2 activity likely results from the reduction of the PQ pool by soluble reductants that accumulate when respiration is blocked. In the absence of PS1 and of a complex I-like enzyme (which may function in a reverse mode), $NAD(P)^+$ reduction by PQH_2 is not likely to occur. NAD(P)H accumulation in the light in presence of myxothiazol and SHAM would then be explained by a competition between NAD(P)H and PS2 for the reduction of PQ, O_2 uptake occurring in thylakoid membranes (chlororespiration).

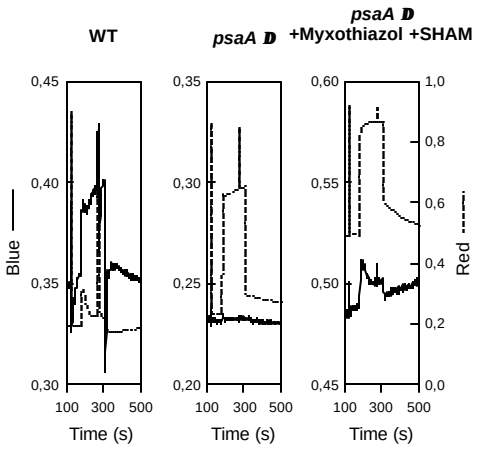

Fig 3: Evolution of chlorophyll ('Red') and NAD(P)H ('Blue') fluorescences during dark-light-dark transitions in WT and PS1-deficient *Chlamydomonas* mutants (*psa*AΔ). On the right figure, cells were incubated in the presence of myxothiazol (4 µM) and SHAM (0.8 mM) before measurements. Light saturating flashes were applied 1 min before and 2 min after light onset.

In higher plant chloroplasts, a NDH complex encoded by plastidial genes has been characterized (5-7). In order to determine the role of the NDH complex during photosynthesis, targeted inactivation of a plastidial *ndh* gene (*ndh*B) was carried out by plastid transformation of tobacco protoplasts. *Ndh*B inactivated tranformants ($ndhB^-$) grew normally and could not be distinguished from WT controls. Western analysis showed that the *ndh*H gene product was absent from thylakoid membranes of $ndhB^-$ (not shown) indicating that the NDH complex disappeared in response to *ndh*B inactivation as previouly reported for other *ndh* genes (7). Chlorophyll fluorescence measurements showed no obvious differences between WT and $ndhB^-$ during a dark to

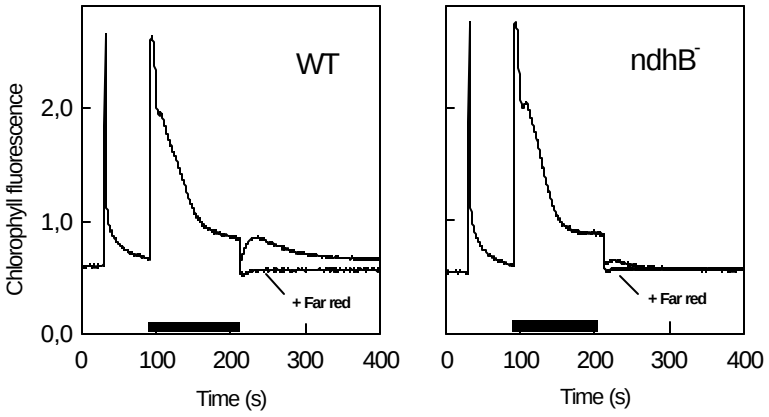

Fig 4 : Chlorophyll fluorescence measurement during a dark to light transition in control and ndhB⁻ tobacco leaves. Light intensity during the light period (–) was 280 µE. $m^{-2}.s^{-1}$. Far red light was switched on after turning the actinic light off in experiments indicated by the arrow.

light transition (Fig. 4). However, in the dark period following illumination, the re-reduction of the PQ pool (which is reverted in the presence of far-red light) was almost completely suppressed in ndhB⁻, thereby indicating that the NDH complex is functional *in vivo*.

Non-photochemical reduction of the PQ pool was studied in tobacco chloroplasts by measuring the effect of an exogenous supply of NADH or NADPH on the chlorophyll fluorescence level measured under non-actinic light (8). Under anaerobic conditions, the NADH (or NADPH)-induced fluorescence rise results from the non-photochemical reduction of the PQ pool (8). In order to determine whether the NDH complex is involved in this reaction, this experiment was performed using chloroplasts prepared from ndhB⁻ leaves. Fig. 5 (A,B) clearly shows that the NADH-induced reduction of the PQ pool is not diminished in ndhB⁻, and seems even enhanced.

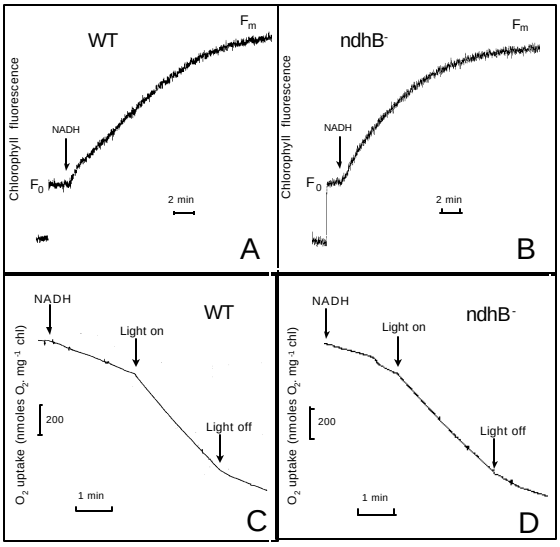

Fig 5 (A,B) : Effect of 2mM NADH addition on the chlorophyll fluorescence level measured in dim modulated light on tobacco chloroplasts. Measurements were performed under anaerobic conditions (in the presence of glucose, glucose oxidase and catalase) using chloroplasts prepared from control (A) and ndhB⁻ (B). **(C,D)** : Light-induced O_2 uptake measured in control (C) and ndhB⁻ (D) tobacco chloroplasts. O_2 uptake measurements were performed in the presence of DCMU (10µM), MV (500µM) and NADH (2mM), catalase (1000 units.ml^{-1}) and superoxide dismutase (500 units.ml^{-1}).

Non-photochemical reduction of intersystem electron carriers was also investigated by measuring light-induced O_2 uptake in the presence of DCMU and methyl viologen (MV). In these conditions, PS2 cannot reduce the PQ pool and light-induced O_2 uptake reflects the ability of PS1 to reduce O_2 using intersystem electron donors. Fig. 5 (C,D) clearly shows that this reaction was not affected in ndhB⁻ chloroplasts. These two experiments show that the

NDH complex is not involved in the NADH-dependent reduction of intersystem electron carriers measured in the chloroplast preparation used here. This is in agreement with the conclusion of a previous study performed on potato chloroplasts, where a NAD(P)H-PQ oxidoreductase activity sensitive to NEM and diphenylene-iodonium, but insensitive to rotenone was characterized (8).

4. Discussion

We conclude from these experiments that different pathways of non-photochemical reduction of the PQ pool take place in chloroplasts. In *Chlamydomonas*, the existence of a significant light-dependent H_2 production in DCMU-treated cells as well as the existence of strong interactions between mitochondrial activity and photosynthetic electron transport activity in PS1-deficient mutants show that electrons can enter the PQ pool from stromal donors. Properties of the H_2 production measured in the presence of DCMU (insensitivity to rotenone and piericidin A) argue against the participation of a NDH complex that would be similar to complex I. We rather conclude to the involvement of another NAD(P)H-PQ oxidoreductase activity, which on the basis of the sensitivity to NEM and the insensitivity to rotenone might be similar to that previously reported in potato chloroplasts (8). This would be consistent with the absence of *ndh* genes in the chloroplast genome of *Chlamydomonas*.

In tobacco, inactivation of the plastidial *ndh*B gene leads to a suppression of the dark re-reduction of the PQ pool, which confirms previous report showing that the plastidial NDH complex is functional *in vivo* (7). However, addition of exogenous NADH to tobacco chloroplasts provokes a reduction of the PQ pool which is unaffected in ndhB$^-$ transformants. We conclude that NDH activity might be lost during chloroplast isolation, and that another pathway of PQ reduction using NAD(P)H (and probably similar to the NAD(P)H-PQ oxidoreductase activity previously characterized in potato chloroplasts - see ref. 8) is present. The existence of different pathways of PQ reduction would explain why *ndh* inactivated transformants grow normally and do not show apparent phenotype.

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Chapitre II

Implication du complexe Ndh dans le transfert cyclique des électrons autour du PS I.

Nous avons montré lors du chapitre précédent que les plantes transplastomiques dont le complexe Ndh n'est pas fonctionnel présentent un retard de croissance et une inhibition de l'assimilation photosynthétique dans des conditions où la disponibilité en CO_2 est limitée. Nous avons émis l'hypothèse que le complexe Ndh est impliqué dans des processus bioénergétiques capables de fournir de l'ATP, probablement via les photophosphorylations cycliques. Cet apport en ATP supplémentaire serait particulièrement utile dans des conditions, comme les conditions photorespiratoires, où la demande en ATP est forte.

Dans la suite de notre travail, nous avons cherché à mettre en évidence un rôle du complexe Ndh dans le transfert cyclique des électrons autour du PSI. Nous avons utilisé l'antimycine A, un composé connu pour inhiber le transfert cyclique et la fixation du CO_2 sur des chloroplastes isolés (Herber et al., 1978 ; Woo et al., 1983) ou des protoplastes (Furbank et Horton, 1987). Nous avons étudié l'effet de l'antimycine A en traitant des disques foliaires de tabac débarrassés de leur épiderme inférieur (voir l'article joint Joët et al. (2001) Plant Physiology, 125 : 1919-1929). Nos expériences ont permis de montrer que l'inhibition des capacités photosynthétiques par l'antimycine A est dépendante des conditions photorespiratoires et donc de la demande en ATP. Le mutant déficient en complexe Ndh est beaucoup plus sensible à l'antimycine A que les plantes sauvages, la photosynthèse étant également inhibée dans des conditions non photorespiratoires où la demande en ATP est faible. De ces expériences, nous avons conclu à l'existence de deux voies de transfert cyclique des électrons autour du PSI *in vivo*. L'une de ces voies impliquerait le complexe Ndh et l'autre voie, sensible à l'antimycine A, impliquerait la FQR.

Toutefois, jusqu'à présent de faibles activités de transfert cyclique des électrons autour du PSI ont été détectées *in vivo* par les techniques de spectrométrie photoacoustique ou par

l'étude des cinétiques de re-réduction du P_{700}^{+} (Herbert et al., 1990 ; Malkin et al., 1992 ; Havaux, 1991). Certains auteurs ont proposé que le transfert cyclique des électrons est contrôlé par l'état rédox des transporteurs d'électrons entre les deux photosystèmes ou par l'état rédox du pool stromal de NADPH (Arnon et Chain, 1979 ; Ziem-Hanck et al., 1980 ; Takahama et al., 1981 ; Hosler et Yocum, 1987). Nous avons émis l'hypothèse que ce type de régulation pouvait intervenir *in vivo*, l'activité chlororespiratoire étant susceptible de contrôler l'état rédox des transporteurs d'électrons entre les deux photosystèmes. Pour tester cette hypothèse, nous avons étudié l'effet de faibles concentrations en oxygène sur l'induction de la photosynthèse (voir l'article joint Joët el al. (1998) G. Garab (ed.), Photosynthesis : Mechanisms and effects, Vol III, 1967-1970) et sur les stockages photochimiques liés à l'activité du transfert cyclique des électrons autour du PS I (voir l'article joint Joët et al., soumis). Ces mesures nous ont permis de montrer de fortes activités de transfert cyclique en conditions de microaérobie et de confirmer l'implication du complexe Ndh dans une des voies de transfert cyclique.

A. Increased sensitivity of photosynthesis to antimycin A induced by inactivation of the chloroplast *ndhB* gene. Evidence for a participation of the NADH-dehydrogenase complex to cyclic electron flow around photosystem I

Joët et al. (2001) *Plant Physiology*, **125** : 1919-1929.

Increased Sensitivity of Photosynthesis to Antimycin A Induced by Inactivation of the Chloroplast *ndhB* Gene. Evidence for a Participation of the NADH-Dehydrogenase Complex to Cyclic Electron Flow around Photosystem I[1]

Thierry Joët, Laurent Cournac, Eva M. Horvath[2], Peter Medgyesy[3], and Gilles Peltier*

Commissariat à l'Energie Atomique, Cadarache, Laboratoire d'Ecophysiologie de la Photosynthèse, Département d'Ecophysiologie Végétale et Microbiologie, Bât. 161, F–13108 Saint-Paul-lez-Durance, France (T.J., L.C., G.P.); and Biological Research Center, Hungarian Academy of Science, P.O. Box 521, H–6701 Szeged, Hungary (E.M.H., P.M.)

Tobacco (*Nicotiana tabacum* var Petit Havana) *ndhB*-inactivated mutants (*ndhB*$^-$) obtained by plastid transformation (E.M. Horvath, S.O. Peter, T. Joët, D. Rumeau, L. Cournac, G.V. Horvath, T.A. Kavanagh, C. Schäfer, G. Peltier, P. Medgyesy-Horvath [2000] Plant Physiol 123: 1337–1350) were used to study the role of the NADH-dehydrogenase complex (NDH) during photosynthesis and particularly the involvement of this complex in cyclic electron flow around photosystem I (PSI). Photosynthetic activity was determined on leaf discs by measuring CO_2 exchange and chlorophyll fluorescence quenchings during a dark-to-light transition. In the absence of treatment, both non-photochemical and photochemical fluorescence quenchings were similar in *ndhB*$^-$ and wild type (WT). When leaf discs were treated with 5 μM antimycin A, an inhibitor of cyclic electron flow around PSI, both quenchings were strongly affected. At steady state, maximum photosynthetic electron transport activity was inhibited by 20% in WT and by 50% in *ndhB*$^-$. Under non-photorespiratory conditions (2% O_2, 2,500 μL L^{-1} CO_2), antimycin A had no effect on photosynthetic activity of WT, whereas a 30% inhibition was observed both on quantum yield of photosynthesis assayed by chlorophyll fluorescence and on CO_2 assimilation in *ndhB*$^-$. The effect of antimycin A on *ndhB*$^-$ could not be mimicked by myxothiazol, an inhibitor of the mitochondrial cytochrome bc_1 complex, therefore showing that it is not related to an inhibition of the mitochondrial electron transport chain but rather to an inhibition of cyclic electron flow around PSI. We conclude to the existence of two different pathways of cyclic electron flow operating around PSI in higher plant chloroplasts. One of these pathways, sensitive to antimycin A, probably involves ferredoxin plastoquinone reductase, whereas the other involves the NDH complex. The absence of visible phenotype in *ndhB*$^-$ plants under normal conditions is explained by the complement of these two pathways in the supply of extra-ATP for photosynthesis.

During oxygenic photosynthesis of C_3 plants, both photosystem II (PSII) and photosystem I (PSI) cooperate to achieve $NADP^+$ reduction using water as an electron donor and generate a trans-membrane proton gradient driving ATP synthesis. Although $NADP^+$ reduction is recognized to be dependent on the activity of both photosystems through electron transport reactions of the "Z" scheme (Hill and Bendall, 1960; Redding et al., 1999), it has early been reported from studies on isolated thylakoids that ATP could be produced by the sole PSI through cyclic electron transfer reactions (Arnon, 1959). The cyclic electron flow around PSI has been extensively studied in thylakoids and/or chloroplasts of C_3 plants (for review, see Fork and Herbert, 1993; Bendall and Manasse, 1995). This mechanism has been suggested to provide ATP for a variety of cellular processes, including stress adaptation (Havaux et al., 1991) and CO_2 fixation (Furbank and Horton, 1987; Herbert et al., 1990). During photosynthetic CO_2 fixation, both NADPH and ATP are used to regenerate ribulose-1,5-bisphosphate and allow functioning of the photosynthetic carbon reduction cycle (Calvin cycle). In the absence of Q cycle, when one NADPH is produced by linear electron transport reactions, four H^+ are released in the lumen. If we consider that translocation of three H^+ is required for the synthesis of one ATP (Hangarter and Good, 1982), the ATP to NADPH ratio produced during linear electron transport would be around 1.33. However in C_3 plants, the ATP to NADPH ratio required for CO_2 fixation has been reported to vary from 1.5 to 1.66, depending on the activity of photorespiration (Osmond, 1981). Insufficient ATP consequently would be synthesized for carbon reduction (Heber and Walker, 1992) and different mechanisms, including cyclic electron flow around PSI, have been proposed to fulfill this func-

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[2] Present address: Department of Genetics, Trinity College, University of Dublin, Dublin 2, Ireland.

[3] Present address: Department of Biology, National University of Ireland, Maynooth, County Kildare, Ireland.

* Corresponding author: e-mail gilles.peltier@cea.fr; fax 33–4–42256265.

tion. A central question is the possible involvement of the Q-cycle, a cyclic electron flow inside the cytochrome (cyt) b_6/f complex (Mitchell, 1975, 1977) able to translocate additional H^+ and therefore provide extra ATP. However, the obligatory character or the flexibility of the Q-cycle during CO_2 fixation remains a matter of debate (Davenport and McCarty, 1984; Ort, 1986; Heber and Walker, 1992; Cramer et al., 1996). Other mechanisms, like cooperation with mitochondrial respiration (Krömer, 1995; Hoefnagel et al., 1998) and Mehler reactions (also known as water-water cycle) (Schreiber and Neubauer, 1990) have also been suggested to re-equilibrate the chloroplastic ATP to NADPH ratio by generating extra-ATP, but their contribution during CO_2 fixation remains to be established.

Cyclic electron transfer reactions around PSI have been early reported to be inhibited by antimycin A (Tagawa et al., 1963). Most studies concluding to an involvement of cyclic electron flow during photosynthesis in C_3 plants have been based on the effect of this compound on photosynthetic reactions such as photophosphorylation (Cleland and Bendall, 1992), rereduction of $P700^+$ (Scheller, 1996), CO_2-dependent O_2 evolution (Furbank and Horton, 1987), $^{14}CO_2$ fixation (Heber et al., 1978; Woo, 1983), or chlorophyll fluorescence (Ivanov et al., 1998). It was suggested that inhibition of photosynthetic reactions by antimycin A was related to the involvement of an antimycin A-sensitive ferredoxin plastoquinone reductase activity in cyclic reactions (Moss and Bendall, 1984; Cleland and Bendall, 1992). The actual efficiency of cyclic electron flow in vivo during photosynthesis of C_3 plants is still unclear (Heber et al., 1995a). Photoacoustic measurements, which allow a direct and quantitative measurement of energy storage by cyclic electron flow around PSI in vivo, have been used to show the existence of cyclic electron transfer reactions in C_4 plants, algae, and cyanobacteria (Herbert et al., 1990). However, until now, this technique failed to show significant cyclic activity in C_3 plants (Herbert et al., 1990; Malkin et al., 1992). For the unicellular alga *Chlamydomonas reinhardtii*, Ravenel et al. (1994), by studying the effect of antimycin A and of different inhibitors on photoacoustic measurements, proposed that two pathways are operating in vivo around PSI. One pathway was shown to be sensitive to antimycin A, whereas the other would involve a NAD(P)H dehydrogenase activity (Ravenel et al., 1994). The existence of an antimycin A-insensitive cyclic electron pathways around PSI was also proposed in C_3 plants from experiments performed in vitro (Hosler and Yocum, 1987; Scheller, 1996).

The plastid genome of higher plants contains genes encoding subunits homologous to the proton-pumping NADH: ubiquinone oxidoreductase, a component of the mitochondrial respiratory chain (Ohyama et al., 1986; Shinozaki et al., 1986). An NADH-dehydrogenase complex (NDH) containing some *ndh* gene products recently has been purified from pea and barley thylakoid membranes (Sazanov et al., 1998; Quiles et al., 2000). To elucidate the function of the plastidial NDH complex in C_3 plants, *ndh* genes were inactivated by chloroplast transformation of tobacco (*Nicotiana tabacum* var Petit Havana) in different laboratories. Inactivation of *ndhB*, *ndh*C, *ndh*K, and *ndh*J genes revealed that the NDH complex is dispensable for plant growth under standard conditions (Burrows et al., 1998; Shikanai et al., 1998; Horvath et al., 2000). The absence of a transient postillumination increase in chlorophyll fluorescence in all NDH-inactivated plastid transformants led to conclude that the NDH complex is involved in the dark reduction of the plastoquinone (PQ) pool, this phenomenon being considered as an after effect of cyclic electron flow around PSI (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). Horvath et al. (2000) recently reported an enhanced growth retardation in $ndhB^-$ inactivated plants when grown under controlled conditions of decreased air humidity. Under such conditions, moderate stomatal closure lowers internal CO_2 concentration, thus increasing the activity of photorespiration. It was proposed by the authors that the NDH complex is involved, via the activity of cyclic electron flow around PSI, in the production of extra-ATP necessary to fulfill the higher ATP demand occurring under photorespiratory conditions.

The aim of the present work is to further study the physiological function of the plastidial NDH complex in plants. For this purpose, we investigated the effect of antimycin A on *ndhB* inactivated plants (Horvath et al., 2000). We observe an increased sensitivity to antimycin A of $ndhB^-$ mutants, this effect being dependent on the photorespiration rate. We conclude to the existence of two cyclic electron transport pathways operating in vivo around PSI, both of these pathways participating to the supply of extra-ATP for photosynthesis.

RESULTS

Chlorophyll fluorescence was measured during dark to light transitions on stripped tobacco leaf discs of WT and $ndhB^-$. In the dark, the nonactinic modulated light allows to determine the F_0 fluorescence level (Fig. 1). Maximal efficiency of PSII was 0.78 ± 0.02 and was similar in both WT and $ndhB^-$. Upon illumination (230 μmol photons m^{-2} s^{-1}), the chlorophyll fluorescence level transiently increased in both WT and $ndhB^-$ and then rapidly decreased due to both photochemical and non-photochemical quenchings. Saturating pulses were used to evaluate photochemical (qP) and non-photochemical (qN) quenching values (Fig. 2). Under illumination, chlorophyll fluorescence induction was similar in WT and $ndhB^-$ (Figs. 1A and 2A). However, a significant difference between the WT and $ndhB^-$ was observed

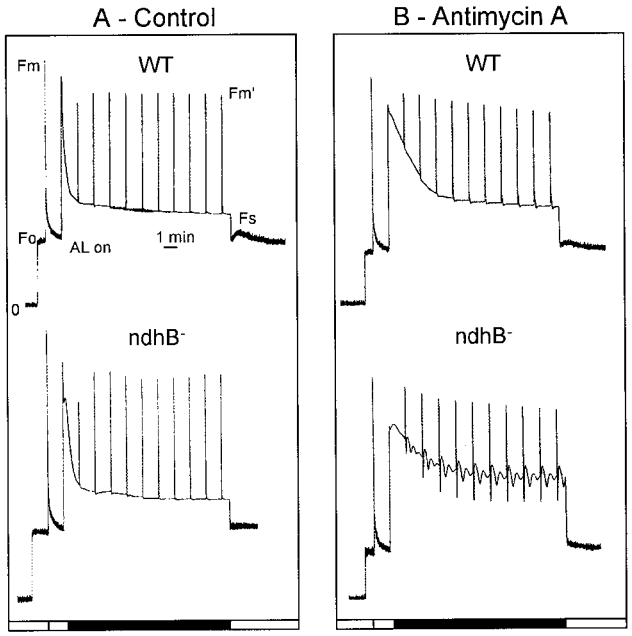

Figure 1. Effect of antimycin A on chlorophyll fluorescence induction curves measured on stripped leaf discs of WT and *ndhB*$^-$ tobacco plants. A, WT and *ndhB*$^-$ in the absence of treatment. B, WT and *ndhB*$^-$ treated with 5 μM antimycin A. Light intensity was 230 μmol photons m^{-2} s^{-1} during the actinic light period (shown by a black box on the *x* axis).

when turning the light off (Fig. 1A). In the WT, a transient increase in the fluorescence level was observed before reaching the F_0 level, this effect being absent in *ndhB*$^-$. This confirms recent work reporting that the postillumination fluorescence rise is absent in *ndh* inactivated mutants (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). Figure 2A shows that qP rapidly increased during the first 2 min of illumination in both WT and *ndhB*$^-$ before reaching progressively a plateau. On the other hand, qN transiently increased after switching on the light and then decreased to a plateau. No significant differences could be detected in qP and qN values between WT and *ndhB*$^-$.

After treatment with antimycin A (Fig. 1B) the maximal efficiency of PSII was not altered (F_v/F_m = 0.78 ± 0.02 in both WT and *ndhB*$^-$), but the chlorophyll fluorescence transient observed following illumination was strongly affected. The fluorescence level F_s of *ndhB*$^-$ remained at a higher value than the WT and quite noticeably, saturating pulse induced strong oscillations of F_s in *ndhB*$^-$ leaves (Fig. 1B). The period of the oscillations was between 20 and 30 s. Quenching analysis was performed during oscillations by illuminating the sample with saturating pulses. Determination of qN and qP values clearly show that oscillations in F_s (Fig. 3A) are due to changes in qP, qN values remaining remarkably stable (Fig. 3B).

As shown in Figure 2B, the transient increase in qN was suppressed by antimycin A in both WT and *ndhB*$^-$, and the qN value progressively reached a level close to that measured in Figure 2A. The establishment of qP was delayed by the antimycin A treatment in WT, but qP finally reached a plateau close to that measured in the absence of antimycin A. The effect of antimycin A was more drastic on *ndhB*$^-$. At steady state, differences between fluorescence induction curves of WT and *ndhB*$^-$ treated by antimycin A (Fig. 1B) were mainly explained by differences in qP values, qN values being less affected (Fig. 2B). Similar effects were observed at high light intensity (1,250 μmol photons m^{-2} s^{-1}; data not shown).

Antimycin A is known to inhibit cyclic electron transport in chloroplasts (Woo, 1983) but is also a potent inhibitor of the cytochrome bc_1 complex in mitochondria. To determine whether the effect of

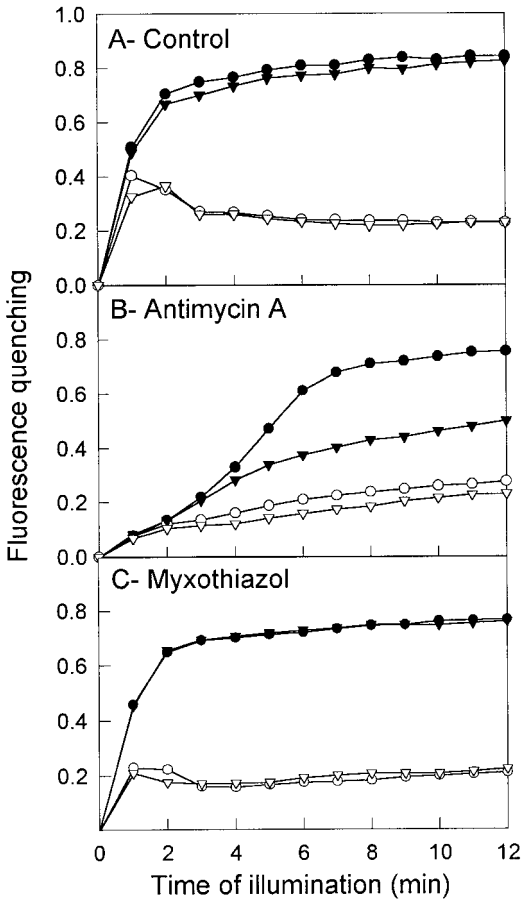

Figure 2. Effect of antimycin A and myxothiazol on photochemical (qP) and non-photochemical (qN) quenchings values during a light to dark transition in WT and *ndhB*$^-$ tobacco. Fluorescence quenchings were measured on stripped leaf discs: A, control; B, treated with 5 μM antimycin; C, treated with 10 μM myxothiazol. WT, ● and ○; *ndhB*$^-$ mutant, ▼ and ▽. Light intensity was 230 μmol photons m^{-2} s^{-1}. Fluorescence levels F_t, F_m, $F_{m'}$, F_0, and $F_{0'}$ were measured during illumination and were used to determine photochemical (qP, black symbols) and non-photochemical (qN, white symbols) quenchings.

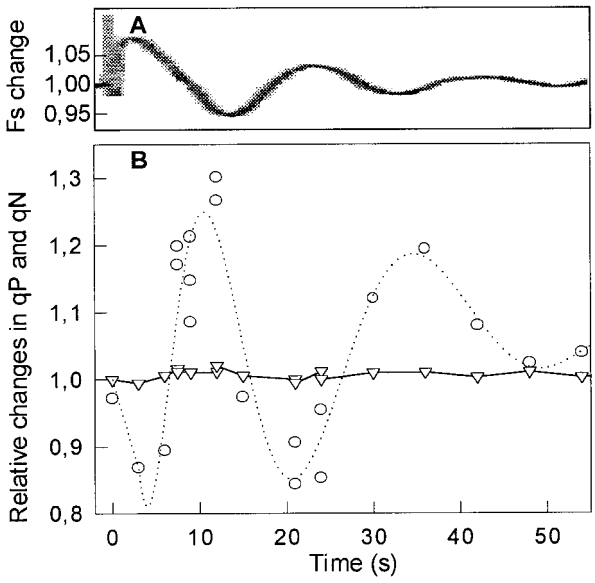

Figure 3. Quenching analysis of light pulse-induced chlorophyll fluorescence oscillations in antimycin A-treated *ndhB*$^-$ leaves. A, Oscillations of F_s induced by a saturating light pulse. B, qP (○) and qN (▽) values were determined during oscillations of F_s by illuminating the sample at various times by a second saturating light pulse. F_s, qP, and qN values are expressed relatively to initial values. Experimental conditions are similar to the experiment shown in Figure 1B.

antimycin A could be attributed to an effect on chloroplasts or mitochondria, we used myxothiazol, another cytochrome bc_1 inhibitor that inhibits the mitochondrial complex by interacting with cytochrome *b* at a different site (von Jagow and Engel, 1981; Thierbach and Reichenbach, 1981). We found that, in contrast to the effect of antimycin A, myxothiazol had no significant effect on qP and a slight inhibitory effect on qN induction curves, this effect being similar in WT and *ndhB*$^-$ (Fig. 2C).

The light saturation of the photosynthetic electron transport was determined at steady state (Fig. 4). In the absence of treatment, both WT and *ndhB*$^-$ leaf discs showed similar light saturation curves. In WT, the antimycin A treatment decreased the maximum photosynthetic electron transport activity by approximately 15%. This decrease was much more pronounced in *ndhB*$^-$ leaf discs (approximately 50%). At high-light intensities, the effect of myxothiazol on WT photosynthesis was similar to the effect of antimycin A (approximately 15% inhibition). However, in contrast to antimycin A, myxothiazol did not generate an additional effect on *ndhB*$^-$ leaf discs photosynthesis (Fig. 4B). It is interesting that at low and medium light intensities (less than 500 μmol photons m^{-2} s^{-1}), myxothiazol had no significant effect on the photosynthetic electron transport activity (Fig. 4B), whereas an inhibitory effect of antimycin A was observed on WT samples under these conditions (Fig. 4A). We checked that under our experimental conditions both antimycin A and myxothiazol inhibited the cyt *bc* pathway of mitochondrial respiration. Respiration rates were measured as CO_2 production in the dark on control leaf discs and on leaf discs treated with respiratory inhibitors (Fig. 5). Antimycin A or myxothiazol alone inhibited the respiration rate by respectively 34% and 16%. Salicyl hydroxamic acid (SHAM), an inhibitor of the alternative oxidase, had almost no effect on respiration. Simultaneous addition of myxothiazol and SHAM or antimycin A and SHAM inhibited the respiration rate by 73% and 83%, respectively, thus showing the participation of the alternative oxidase pathway. Note that when added in the presence of antimycin A, myxothiazol, or alone, SHAM had no effect on chlorophyll fluorescence induction curves (data not shown). As antimycin A and myxothiazol similarly inhibited mitochondrial respiration, we conclude that the additional effect of antimycin A on *ndhB*$^-$ leaf discs compared with the WT is not related to a mitochondrial inhibition but rather linked to the inhibition of a chloroplast process.

The effect of antimycin A on photosynthesis was investigated under different photorespiratory conditions, by simultaneously measuring at steady-state

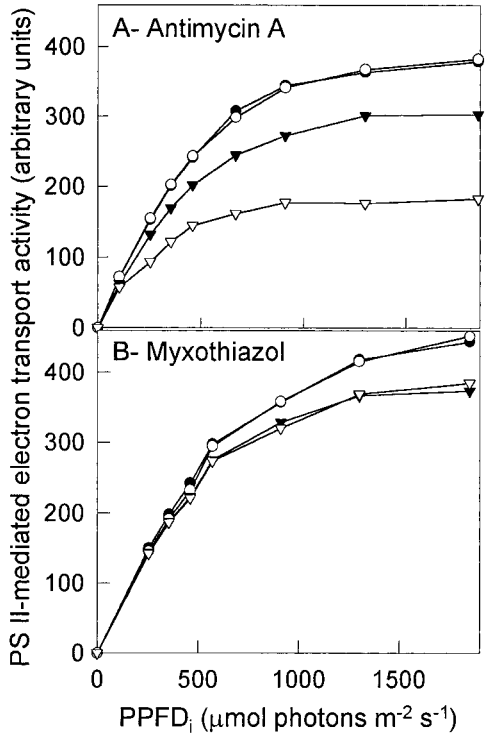

Figure 4. Photosynthetic electron flow estimated as ($\Delta F/F_{m'} \times PPFD_i$) as a function of light intensity (incident PPFD) in stripped leaf discs treated with antimycin A or myxothiazol. Stripped tobacco leaf discs were treated with 5 μM antimycin or 10 μM myxothiazol. ●, WT control; ○, *ndhB*$^-$ control; ▼, WT treated with 5 μM antimycin A (A) or 10 μM myxothiazol (B); ▽, *ndhB*$^-$ treated with 5 μM antimycin A (A) or 10 μM myxothiazol (B).

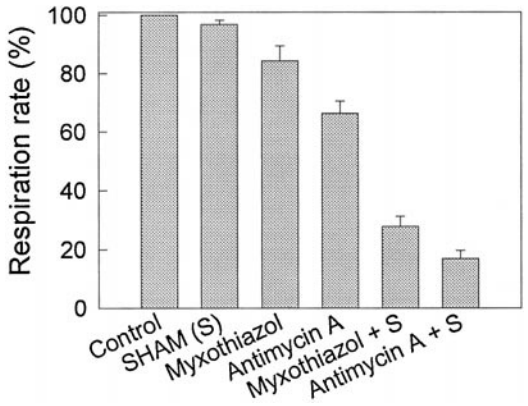

Figure 5. Inhibition of tobacco leaf discs respiration by antimycin A (5 μM), myxothiazol (10 μM), and SHAM (0.6 mM). WT tobacco leaf discs were stripped and treated with inhibitors. Respiration rates were measured by following CO_2 production in the dark. Values are the average of three independent measurements. Vertical bars represent SEs.

PSII-mediated electron transport activity and CO_2 assimilation. In air, relative inhibition by antimycin A of PSII activity was approximately 17% for the WT and 33% for ndhB$^-$ leaves (Fig. 6A). Similar effects of antimycin A were observed on CO_2 assimilation, although differences between WT and *ndhB*$^-$ appeared less obvious. Under non-photorespiratory conditions (2% [v/v] O_2, 2,500 μL L^{-1} CO_2), where the ATP demand is decreased (Osmond, 1981), photosynthetic activity of WT leaves was almost unaffected by antimycin A (less than 3% inhibition at the steady state; Fig. 6B). In contrast, in ndhB$^-$ leaves relative inhibition by antimycin A was similar to that observed in air (approximately 25% inhibition).

DISCUSSION

Involvement of the NDH Complex in Cyclic Electron Flow around PSI

We observed a sensitivity of photosynthesis to antimycin A in tobacco leaves (monitored either by chlorophyll fluorescence or gas exchange measurements), which was increased in *ndhB*$^-$ mutants. Sensitivity of the electron transport to antimycin was highest at saturating light intensity and so was the difference between WT (20% inhibition) and mutant (50% inhibition). The additional inhibitory effect observed in *ndhB*$^-$ mutants was not observed when using myxothiazol, thus showing that it is related to an inhibition of plastidial rather than mitochondrial reactions.

In addition to its well-known effect on cyclic electron flow, antimycin A has been reported to affect qN (Oxborough and Horton, 1987; Ivanov et al., 1998). In our experiments, antimycin A induced a significant delay in the establishment of qN, but the steady-state level was virtually not affected and no differences were observed between WT and *ndhB*$^-$ even at high intensities, when qN reaches its maximal values. In antimycin-treated leaves, F_s values at steady state remained higher in mutants than in WT, whereas the qN values were comparable. This higher F_s in mutants was then not related to variations in qN, but attributable to a more reduced state of the plastoquinone pool, indicating a less efficient functioning of electron acceptor reactions after PSI (Calvin cycle, etc.). We conclude that the simultaneous inhibition by antimycin A of cyclic electron flow around PSI and of NDH activity by gene inactivation leads to a reduced ability to use reducing power on the acceptor side of PSI.

Previous studies, based on the disappearance of the transient postillumination rereduction of PQ in *ndh*-inactivated mutants (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998) or on a decrease of the P700$^+$ reduction rate in the dark (Burrows et al., 1998) already concluded to an involvement of the NDH complex in intersystem chain reduction and therefore to its potential implication during cyclic electron transport. It appears from our experiments that the NDH activity is involved in cyclic electron transport together with the antimycin-sensitive pathway. Since photosynthesis is only slightly inhibited by antimycin A in WT, we conclude that the NDH-mediated pathway has a sufficient efficiency to compensate for the antimycin-sensitive pathway to a large extent.

The existence of different cyclic electron pathways around PSI has previously been suggested in the literature. In spinach thylakoids, Hosler and Yocum (1985) reported the insensitivity to antimycin A of cyclic photophosphorylations measured in the presence of ferredoxin and NADP$^+$. Based on photoacoustic measurements performed in vivo in *C. reinhardtii* cells, Ravenel et al. (1994) observed that antimycin A and *N*-ethyl-maleimide could inhibit PSI energy storage in vivo when added together, these compounds having no effect when added alone. More recently, based on P700$^+$ rereduction measurements performed in barley thylakoids, Scheller (1996) proposed the existence of an antimycin-insensitive cyclic electron transport around PSI. The involvement of the NDH complex in cyclic electron flow in association with other pathways was shown in cyanobacteria (Mi et al., 1992; Yu et al., 1993) and recently suggested in higher plants from in vitro experiments performed on broken chloroplasts (Endo et al., 1998). Based on a differential sensitivity to antimycin A of PQ reduction in the WT and in a *ndhB*$^-$ mutant, these authors concluded to the existence of two pathways, one of them involving the NDH complex. All of the evidences obtained in C_3 plants are based on experiments performed on in vitro systems. Our study, performed on leaves, clearly shows the importance of cyclic pathways during photosynthesis in C_3 plants in vivo.

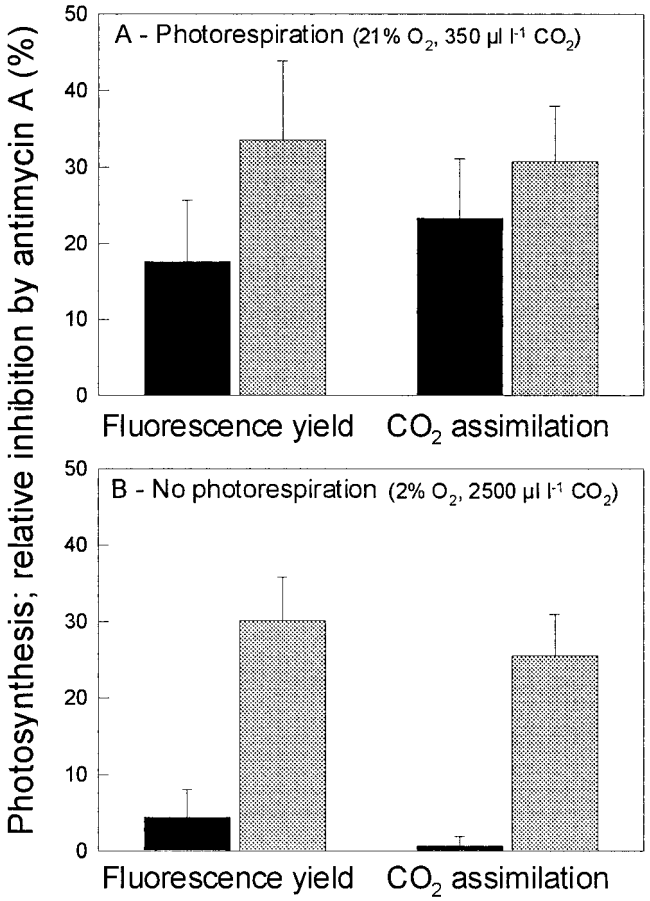

Figure 6. Inhibition of photosynthesis by antimycin A measured on stripped leaf discs of WT (black square) and *ndhB*$^-$ (gray square) mutant. Photosynthesis was measured by following the chlorophyll fluorescence yield ($\Delta F/F_{m'}$) or by measuring CO_2 assimilation rates. A treatment with 5 μM antimycin A was performed under a light intensity of 100 μmol photons m^{-2} s^{-1}. Values are the average of eight independent measurements performed on four independent experiments. Vertical bars represent SEs.

Antimycin A was found to induce strong damped oscillations of the fluorescence yield in response to saturating pulse. This effect, observed in *ndhB*$^-$ mutant leaves but not in WT, was attributed to variations in the redox level of Q_A, since no variation in qN could be detected during oscillations. Oscillations in chlorophyll fluorescence yield and in O_2 evolution rates have been previously reported to be induced by rapid changes in light intensity or gas composition (Slovacek et al., 1980). They have been proposed to result from an imbalance between ATP production (by either linear or cyclic electron transport) and ATP consumption (by photosynthetic carbon reduction or oxidation cycle) processes occurring in response to rapid changes in light intensity or gas composition (Furbank and Horton, 1987; Horton and Nicholson, 1987; Veljovic et al., 1990). Based on the effect of antimycin A on O_2 evolution and chlorophyll fluorescence observed in barley protoplasts during light transitions, and particularly on the fact that antimycin A increased the frequency of oscillations, it was concluded that cyclic electron flow is involved in the ATP balance during the early phase of illumination (Quick and Horton, 1985; Furbank and Horton, 1987). In our conditions, inactivation of the NDH complex induced strong oscillations of chlorophyll fluorescence in antimycin A-treated leaves, therefore suggesting that the NDH complex is involved in the supply of ATP for photosynthesis.

The differential effect of antimycin A on WT and *ndhB*$^-$ could be observed using antimycin A concentrations as low as 1 μM (data not shown), whereas optimal effects were obtained at 5 μM. At medium light intensities (less than 500 μmol photons m^{-2} s^{-1}) inhibition of photosynthetic electron transport by antimycin A was approximately 15%. The inhibition of photosynthesis by antimycin A measured using in vitro systems such as thylakoids or chloroplasts generally reported in the literature is much more important, generally between 50% and 80% (Tagawa et al., 1963; Mills et al., 1978; Woo, 1983; Moss and Bendall, 1984; Cleland and Bendall, 1992). We observed a much more important inhibition of the photosynthetic electron transport activity in *ndhB*$^-$ plants (approximately 50%). One possible explanation for the hypersensitivity of in vitro systems to antimycin A may be the lability of the NDH complex during organelle isolation procedures (Guedeney et al., 1996; Sazanov et al., 1998) or dysfunctioning of the NDH complex under in vitro conditions. It is interesting that Hosler and Yocum (1985) reported particular conditions where photophosphorylation measured in spinach thylakoids membranes system with ferredoxin and $NADP^+$ was not sensitive to antimycin A.

Our experiments highlight the importance of cyclic electron transport during photosynthesis in C_3 plants in vivo. This point has been a matter of debate in the last decade given that direct measurements of cyclic electron flow such as those given by photoacoustic experiments failed to detect any significant activity in C_3 plants (Heber and Walker, 1992; Bendall and Manasse, 1995). Using photoacoustic measurements on peas, Malkin et al. (1992) measured a weak cyclic activity, saturating at low-light intensity. In the same way, based on measurements of P700 rereduction, chlorophyll fluorescence, and light scattering on spinach, Heber et al. (1995a) concluded to low cyclic activity in C_3 plants, mainly restricted to the control of PSII, taking part in the complex machinery that acts to protect the photosynthetic apparatus against photo-inhibition. In contrast, and in line with our present data, a recent study by Cornic et al. (2000) based on $P700^+$ rereduction and light scattering measurements, performed on pea and spinach leaves, concluded that cyclic electron flow around PSI participates to the ATP supply during photosynthesis. The apparent discrepancy between conclusions based on photoacoustic measurements and those ob-

tained using antimycin A and/or mutations is an intriguing question that remains to be answered.

ATP Supply, Cyclic Electron Flow, and Photorespiration

At low-light intensity and under non-photorespiratory conditions, antimycin A had no significant effect on the steady-state photosynthesis rate of WT. Under such conditions, the ATP to NADPH ratio required for CO_2 fixation is only 1.5 (Osmond, 1981) and the NDH complex pathway likely provides sufficient extra-ATP to reach optimal photosynthesis rates. Under the same conditions, the significant effect of antimycin A observed in the *ndhB*$^-$ mutant (25% inhibition rate), which was not mimicked by myxothiazol, shows that under the lowest ATP demand, a minimal activity of cyclic electron flow is required. As a consequence, we conclude that the Q-cycle is not able to fully satisfy the ATP demand in these conditions. Under photorespiratory conditions, like in air, needs for extra-ATP are increased (Osmond, 1981). In such conditions, antimycin A significantly inhibited electron transport in WT and an enhanced effect was observed in *ndhB*$^-$ mutants. This likely reflects the fact that the sole NDH-mediated pathway is unable to fully satisfy the ATP demand. These interpretations are consistent with data previously reported on the same tobacco *ndhB*$^-$ mutant by Horvath et al. (2000). These authors reported significant growth retardation when growing *ndhB*$^-$ plants under CO_2 limitation occurring in response to a moderate water limitation or abscisic acid spraying. These conditions induce stomatal closure and consequently reduce internal CO_2 concentration, thus stimulating the photorespiration rate (Cornic and Briantais, 1991; Lawlor, 1995). We therefore conclude, in agreement with Horvath et al. (2000), that the NDH complex is involved in extra-ATP supply under conditions where photorespiration is high. The NDH complex recently was proposed to be involved in photoprotection (Endo et al., 1999). The sensitivity of a *ndhB*$^-$ mutant to photo-inhibition was explained by an involvement of the NDH complex in the control of electron flow through PSII, which may be mediated by pH changes. Noticeably, photorespiration has been proposed to protect C_3 plants from photooxidation and to prevent photo-inhibition (Heber et al., 1995b; Kozaki and Takeba, 1996). Therefore, a possible role of the NDH complex in producing extra-ATP necessary to sustain high photorespiration rates should also be considered to explain the higher sensitivity of *ndhB*$^-$ to photo-inhibition.

Involvement of Other Mechanisms for Extra-ATP Supply and H^+ Requirement for ATP Synthesis

One of the central questions concerning the debate about the extra-ATP supply for photosynthesis is how the cytochrome $b_{6/f}$ complex mediates the oxidation of plastoquinol. In case the Q-cycle would be obligatory during the "Z" scheme, electron shuttled back to the plastoquinone pool through the Mitchellian Q-cycle would increase the H^+ gradient and in turn form more ATP (Davenport and McCarty, 1984; Rich, 1991). If we consider that three H^+ are needed to synthesize one ATP, there would be no need for other mechanisms to supply extra-ATP for CO_2 fixation (Rich, 1988). However, if the H^+/ATP ratio is four, as reported by Kobayashi et al. (1995) and Rumberg et al. (1990), other mechanisms of extra ATP supply would be needed. Another thing to consider is that, assuming that both Suc and starch are the predominant end products of photosynthesis, there is an additional cost for CO_2 fixation of 0.17 mol ATP per mol CO_2 fixed, consumed in the formation of glycosidic bonds (Furbank et al., 1990). Results presented here provide evidence that an input from cyclic electron transport is essential to fully satisfy the ATP requirements of C_3 plants. This is in accordance with recent studies of Cornic et al. (2000) concluding to the involvement of both cyclic electron flow around PSI and of the Q-cycle for the supply of ATP.

At high-light intensity, maximum photosynthetic electron transport rates measured in WT were inhibited by approximately 20% indistinctly by antimycin or myxothiazol. This effect, which is clearly related to a mitochondrial inhibition, might be explained by a cooperation between chloroplasts and mitochondria to achieve maximal photosynthetic rates. Two mechanisms of interaction can be proposed to explain such a dependency. In the first one, Gly decarboxylation, occurring in mitochondria during the photosynthetic carbon oxidation cycle (or photorespiration), produces NADH. Inhibition of the mitochondrial respiratory chain, by preventing NADH oxidation, might explain such an inhibition. In the second one, part of reducing equivalents produced in the chloroplast during photosynthesis might be shuttled to mitochondria. After mitochondrial conversion to ATP, shuttling back to chloroplasts might participate to re-equilibrate the chloroplastic ATP to NADPH ratio. Such a mechanism was proposed by Krömer (1995), based on the inhibition of photosynthesis by mitochondrial inhibitors like oligomycin in protoplasts. Whatever the mechanism involved in this interaction, it is interesting to note that the mitochondrial contribution is almost undetectable at low light intensity (below 400 μmol photons m^{-2} s^{-1}). We therefore propose that mitochondrial contribution to ATP supply, if it occurs, acts as an ultimate mechanism, which may be used when other mechanisms such as Q-cycle, cyclic pathways are already fully engaged in ATP production.

CONCLUSION

In conclusion, differences in steady-state photosynthetic activities could be observed between WT and

ndhB$^-$ mutants when treating leaves with antimycin A, an inhibitor of cyclic electron flow around PSI. These effects are interpreted by the existence of two independent cyclic electron pathways around PSI, one pathway being sensitive to antimycin A and the other, insensitive to antimycin A, involving the plastidial NDH complex. Under non-photorespiratory conditions (CO_2-enriched air), each pathway would be able to support the extra-ATP demand of photosynthetic CO_2 fixation. Under photorespiratory conditions, like in air, the antimycin A-sensitive pathway would be able to provide sufficient extra-ATP, whereas the NDH-dependent pathway alone would be limiting CO_2 assimilation. Under high photorespiration rate (occurring for instance when stomata close in response to a water limitation) both antimycin A-sensitive pathway and NDH complex are needed to re-equilibrate the chloroplastic ATP to NADPH ratio, thus explaining why *ndhB*$^-$ mutants grow more slowly than WT in response to a water shortage (Horvath et al., 2000).

MATERIALS AND METHODS

Plant Material and Preparation of Leaf Samples

Wild-type tobacco (*Nicotiana tabacum* var Petit Havana) and *ndhB*-inactivated mutants (Horvath et al., 2000) were grown on compost in a phytotron (25°C day/20°C night; 12-h photoperiod) under a light fluence of 350 μmol photons m^{-2} s^{-1} supplied by quartz halogen lamps (HQI-T 400W/DV, Osram, Germany). Plants were watered using a half-diluted nutritive solution (Hoagland and Arnon, 1950). Leaf discs (12-mm diameter) were sampled from 5- to 8-week-old plants. After stripping the lower epidermis, leaf samples were kept in the dark on a moist paper filter in a close Petri dish until use. Stripped tobacco leaf discs were soaked in Petri dishes containing water and inhibitors. Times of incubation were respectively 20 and 90 min for photosynthesis and respiration measurements. Inhibitors were added diluted in methanol (maximal final methanol concentration was 0.5% [v/v]). Control leaf discs were soaked in Petri dishes containing water and methanol.

Chlorophyll Fluorescence Measurements

Stripped leaf discs were deposited on a wet filter and placed under a watch glass. Chlorophyll fluorescence was measured using a pulse modulated amplitude fluorometer (PAM-2,000, Heinz-Walz, Effeltrich, Germany). The optic fiber of the fluorometer was in contact with the watch glass. Non-actinic modulated light (655-nm maximum emission, 600 Hz) was used to determine the chlorophyll fluorescence level F_0. Maximum chlorophyll fluorescence level (F_m) was measured following a saturating pulse (0.8-s duration) of white light (10,000 μmol photons m^{-2} s^{-1}). For determination of qP and qN, leaf discs were exposed to actinic light and pulsed every 60 s by a 10,000-μmol photons m^{-2} s^{-1} saturating pulse (0.8-s duration) according to Quick and Stitt (1989). The maximal efficiency of PSII was determined as F_v/F_m (Kitajima and Butler, 1975). Apparent PSII activity under illumination, reflecting the electron transport rate of the photosynthetic chain, was estimated from quantum yield measurement as:

$$(F_{m'}-F_s)/F_{m'} \times PFFD_i \text{ (Genty et al., 1989)}$$

Photosynthetic CO_2 Fixation Measurements

CO_2 exchange measurements were performed using a LICOR LI-6,262 analyzer in a differential mode on stripped tobacco leaf discs kept on a moist paper filter in a homemade chamber. Chlorophyll fluorescence was measured simultaneously using a PAM-2,000 fluorometer as described above. A LICOR LI-610 portable Dew Point Generator was used to generate moist air (75% relative humidity) at a flow rate of 2 mL s^{-1}. A gas mixer (SEMY Engineering, Montpellier, France) was used to generate gas mixtures with various O_2 and CO_2 concentrations. Unless specified, CO_2 concentration was 350 μL L^{-1} and O_2 concentration was 20%. O_2 concentration was monitored by an O_2 analyzer OXOR 6 N (MAIHAK, Hamburg, Germany) and CO_2 concentration using an infrared gas analyzer (LI-6,262, LI-COR, Lincoln, NE).

Respiration Measurements

Five leaf discs (10-mm diameter) were placed on a wet filter paper in the sample chamber of a close gas circuit connected to a UNOR 6 N (MAIHAK) CO_2 analyzer. Respiration was measured at room temperature (20°C) as the CO_2 production rate in the dark.

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B. Increased sensitivity of photosynthesis to anaerobic conditions induced by targeted inactivation of *ndhB* gene

Joët el al. (1998) G. Garab (ed.), *Photosynthesis : Mechanisms and effects*, **Vol III**, 1967-1970.

INCREASED SENSITIVITY OF PHOTOSYNTHESIS TO ANAEROBIC CONDITIONS INDUCED BY TARGETED INACTIVATION OF THE CHLOROPLAST *ndh*B GENE

[1]Thierry Joët, [2]Zoran Cerovic, [1]Dominique Rumeau [1]Laurent Cournac, [1]Geneviève Guedeney, [3]Eva Horváth, [3]Peter Medgyesy, [1]Gilles Peltier

[1]CEA Cadarache, DEVM Bat 161, 13108 Saint-Paul-lez-Durance, France

[2]CNRS-LURE Bat 203, Centre Universitaire Paris-Sud, 91405 Orsay, France

[3]BRC, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

Keywords : alternative electron transport - cyclic electron transfer - NAD(P)H-dehydrogenase - chl fluorescence induction - Mehler reaction - plastid transformation

1. Introduction

The plastid genome of higher plants has been reported to contain 11 *ndh* genes which have been recently shown to encode a NADH dehydrogenase (NDH) complex (1, 2). The NDH complex has been suggested to be involved in the reduction of plastoquinones occurring during cyclic electron flow around PSI (3) or during chlororespiration. Inactivation of *ndh* genes has been achieved by plastid transformation in tobacco (4). Inactivation of *ndh*C, *ndh*K and *ndh*J genes revealed that this complex is functional and catalyzes the post-illumination reduction of the PQ pool (4). However, a possible participation of the NDH complex during light reactions and particularly an involvement in cyclic electron flow was not shown by the authors. It has been previously reported that cyclic electron flow was stimulated under anaerobic conditions (5) due to an adequate redox poise of the NAD(P)/NAD(P)H ratio (6). Also, O_2, by accepting electrons during Mehler reactions, could drain electrons out of the cyclic electron pathway (7). We hypothesized that differences in photosynthetic activity might be observed in *ndh*-disrupted plants in anaerobiosis conditions where cyclic electron flow is expected to be stimulated. Targeted inactivation of the plastidic *ndh*B gene was achieved by plastid transformation of tobacco. Analysis of homoplasmic transformants showed that *ndh*B inactivation leads to a disappearance of the NDH complex. Although no difference in chlorophyll fluorescence was observed between control and inactivated ndhB transformants (ndhB$^-$) during a dark to light transition performed under aerobic conditions, we observed a significant reduction of electron transfer activity under anaerobic conditions in ndhB$^-$ leaves.

2. Procedure

Tobacco leaf discs (10 mm Ø) were sampled from 5 to 8 week-old plants grown in phytotron under a light intensity of 350 µE m^{-2} s^{-1}. After stripping the lower epidermis (limiting gas diffusion), leaf discs were placed in the sample compartment of a modified experimental device for chlorophyll fluorescence and blue-green fluorescence measurements (8). The pulsed excitation light was delivered by a high-power xenon flash lamp (L4633, Hamamatsu) while the actinic light was provided by an array of 7 red light emitting diodes (LED) (HLMP-8150, Hewlett Packard). The

maximum chlorophyll fluorescence level (F_m) was measured under a 0.8 s saturating pulse of white light (800 $\mu E.m^{-2}.s^{-1}$). Photochemical and non-photochemical fluorescence quenchings were determined according to (9). CO_2-free air or pure nitrogen were flushed over the sample at a rate of 45 $L.h^{-1}$.

The *ndh*B gene was inactivated in tobacco (Petit Havana) by replacing the gene with a mutant version possessing a translational stop in the coding region. The mutated gene was physically linked to selectable markers (spectinomycin and streptomycin resistance) located on the same plastid DNA fragment derived from a *Solanum nigrum* plastid mutant. Plastid transformants were produced via polyethylene glycol -mediated transformation of tobacco protoplasts, and selection for the binding-type antibiotic resistances was used to generate single-cell derived homoplasmic primary regenerates of plastids transformants (10). Physical mapping and Southern hybridation were performed to confirm the presence of the mutation in the transformants. Control lines were generated by inserting the selectable markers alone.

3.Results

Chlorophyll fluorescence was measured during a dark to light transition under aerobic (CO_2-free air) and anaerobic (N_2) conditions on stripped leaves of wild-type (WT), control and ndhB$^-$ tobacco.

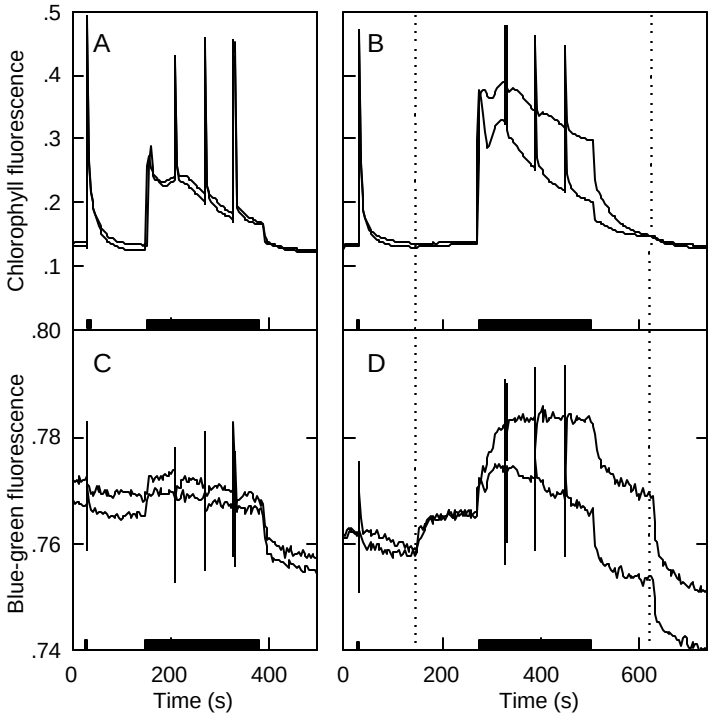

Figure 1. Chlorophyll fluorescence (A,B) and blue-green fluorescence (C,D) measured in tobacco leaf discs during a dark to light transition in the presence of CO_2-free air (A,C) or N_2 (B,D). Control and ndhB$^-$ stripped leaf discs were subjected to anaerobic conditions as indicated by dotted lines. Light intensity was 21 $\mu E.m^{-2}.s^{-1}$ (−−). Control (**thick line**), ndhB$^-$ (thin line). In (C) and (D), a 0.04 offset in the blue-green fluorescence value was applied in controls.

Control transformants and WT showed similar behaviour in all of the experiments described here (not shown). Before induction, a saturating flash was used to determine Fv/Fm the optimal quantum yield of PS II (9) which was found to be around 0.76 and was similar in control and ndhB$^-$. Under aerobic conditions, chlorophyll fluorescence transients were similar in control and ndhB$^-$ (Fig. 1A) while under anaerobic conditions a marked difference was observed (Fig 1B). Saturating flashes were used to determine photochemical and non-photochemical quenchings (Fig. 2).

Under anaerobic conditions, qP rapidly reached a plateau during the first minute of illumination, whereas qN progressively increased during at least 4 minutes. Fig. 2 shows that the difference in fluorescence transients between control and ndhB$^-$ is mainly due to a difference in qP values. Measurements of blue green fluorescence (BGF) were performed in the same experiment to determine changes in the redox state of the NAD(P)/NAD(P)H pool (8). Under aerobic conditions, BGF slightly increased upon illumination reflecting a reduction of the NAD(P) pool (Fig. 1C). No difference was observed between control and ndhB$^-$. In the dark, N_2-flushing induced a similar increase in BGF level in both samples (Fig. 1D). Illumination induced a much stronger increase of BGF in ndhB$^-$ than in control leaves, although the initial increase rate was identical. Quite interestingly, the BGF level was stable during illumination in ndhB$^-$ while it decreased in the control.

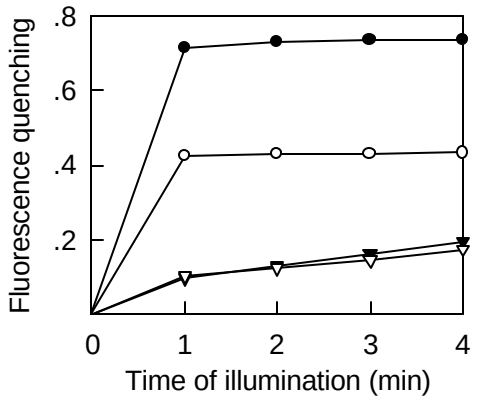

Figure 2. Effect of anaerobiosis on evolution of photochemical (qP) and non-photochemical (qN) quenchings during a dark to light transition in control and ndhB$^-$ tobacco leaf discs. Samples were flushed with N_2 for 2 min before the light was switched on. Ft, Fm, Fm', F0 and F_0' were measured during illumination and were used to determine photochemical quenching (qP) in control (●) and ndhB$^-$ (○), and non-photochemical quenching (qN) in control (♦) and ndhB$^-$ (∇). Light intensity was 25 µE.m^{-2}.s^{-1}.

Effective PS II quantum yield under illumination was estimated from (Fm'-Ft)/Fm' (9) and was measured at different light intensities (Fig. 3A). PS II quantum yield was almost stable in both control and ndhB$^-$ under aerobic conditions. However, under anaerobic conditions, PS II efficiency of both control and ndhB$^-$ gradually decreased, the decrease being much more pronouced in ndhB$^-$. Apparent PS II activity, deduced from the fluorescence yield and estimated from (Fm'-Ft)/Fm' x PPFDi is shown in Fig. 3B. This graphical representation allows to visualize the light saturation of PS II activity. Under anaerobic conditions, photosynthetic electron transport activity of ndhB$^-$ reached a plateau, which was about 40% lower than in the control.

4. Discussion

While no difference in fluorescence induction was observed between control and ndhB$^-$ under aerobic conditions, a clear difference was revealed under anaerobiosis, which was due to a reduced electron transport activity in ndhB$^-$. Under anaerobic conditions (N_2), electron acceptors should be less available, both the Calvin cycle and Mehler reaction activity being strongly reduced (lack of CO_2 and O_2). However, some electron transport activity saturating at low light was observed, suggesting the presence of an electron acceptor. The acceptor might be O_2 produced at PS II, nitrite or an reutilization of NAD(P)H by a low level of Calvin cycle activity. This electron transport activity was more important in control than it ndhB$^-$ (Fig. 3B), At the same time, the redox state of the NAD(P)/NAD(P)H pool initially increased at a similar rate both in ndhB$^-$ and control, but reached a much higher value in ndhB$^-$, suggesting that NAD(P)H is recycled more efficiently in the control.

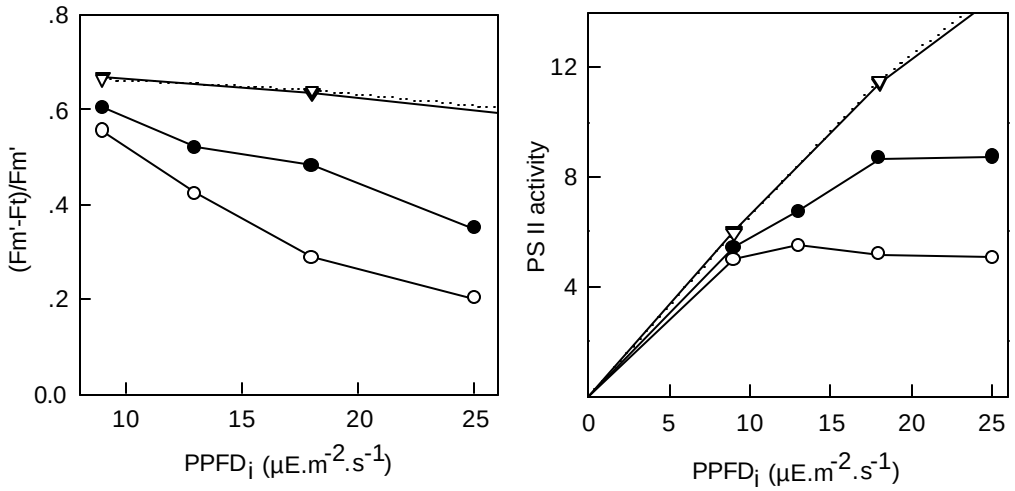

Figure 3. A : PS II quantum yield estimated from ((Fm'-Ft)/Fm') as a function of light (incident photosynthetic photon flux density, $PPFD_i$) in stripped tobacco leaf discs. For anaerobic conditions leaves were flushed with N_2 in the dark during 2 min before light was turned on. Photosynthetic activity was measured 4 min after turning on the light. Anaerobic conditions: control (♦), ndhB$^-$ (●); Control and ndhB- under CO_2-free air (∇;○). **B :** Photosynthetic flow estimated from ($\Delta F/Fm'$ x $PPFD_i$) as a function of light intensity. Anaerobic conditions: Control (♦);ndhB$^-$ (●);Control and ndhB- under CO_2-free air (∇;○).

We interpret these data through an involvement of the NDH complex in cyclic electron flow around PS I. When O_2 is lacking, cyclic electron flow activity would be necessary to activate the reutilization of NAD(P)H, may be through the establisment of a proton gradient. In the presence of O_2, this activation would be achieved by pseudocyclic activity (Mehler reaction), explaining the absence of difference between control and ndhB$^-$. We conclude that the NDH complex is involved in cyclic electron flow. However, under aerobic conditions, pseudocyclic electron flow could replace cyclic electron flow activity which might explain the lack of phenotype in *ndh* inactivated plants.

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C. Cyclic electron flow around PS I in C_3 plants: *in vivo* control by the redox state of chloroplasts and involvement of the Ndh complex

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Cyclic Electron Flow around Photosystem I in C_3 Plants. In Vivo Control by the Redox State of Chloroplasts and Involvement of the NADH-Dehydrogenase Complex

Thierry Joët, Laurent Cournac, Gilles Peltier, and Michel Havaux*

Commissariat à l'Energie Atomique/Cadarache, Département d'Ecophysiologie Végétale et de Microbiologie, Laboratoire d'Ecophysiologie de la Photosynthèse, Unité Mixte de Recherche 163 Centre National de la Recherche Scientifique/Commissariat à l'Energie Atomique, Univ-Méditerranée/Commissariat à l'Energie Atomique 1000, F–13108 Saint-Paul-lez-Durance, France

Cyclic electron flow around photosystem (PS) I has been widely described in vitro in chloroplasts or thylakoids isolated from C_3 plant leaves, but its occurrence in vivo is still a matter of debate. Photoacoustic spectroscopy and kinetic spectrophotometry were used to analyze cyclic PS I activity in tobacco (*Nicotiana tabacum* cv Petit Havana) leaf discs illuminated with far-red light. Only a very weak activity was measured in air with both techniques. When leaf discs were placed in anaerobiosis, a high and rapid cyclic PS I activity was measured. The maximal energy storage in far-red light increased to 30% to 50%, and the half-time of the P_{700} re-reduction in the dark decreased to around 400 ms; these values are comparable with those measured in cyanobacteria and C_4 plant leaves in aerobiosis. The stimulatory effect of anaerobiosis was mimicked by infiltrating leaves with inhibitors of mitochondrial respiration or of the chlororespiratory oxidase, therefore, showing that changes in the redox state of intersystem electron carriers tightly control the rate of PS I-driven cyclic electron flow in vivo. Measurements of energy storage at different modulation frequencies of far-red light showed that anaerobiosis-induced cyclic PS I activity in leaves of a tobacco mutant deficient in the plastid Ndh complex was kinetically different from that of the wild type, the cycle being slower in the former leaves. We conclude that the Ndh complex is required for rapid electron cycling around PS I.

During oxygenic photosynthesis, photosystem (PS) II and PS I cooperate to achieve a linear electron flow from H_2O to $NADP^+$ and to generate a transmembrane proton gradient driving ATP synthesis. However, ATP can also be produced by the sole PS I through cyclic electron transfer reactions (Arnon, 1959). This mechanism enables the generation of a proton gradient across the thylakoid membrane without NADP reduction by rerouting electrons of reduced PS I acceptors toward the intersystem carriers. Cyclic and linear electron transfers share a common sequence of electron carriers, namely the plastoquinone (PQ) pool, cytochrome b_6/f complex, and plastocyanin (for review, see Fork and Herbert, 1993; Bendall and Manasse, 1995). This alternative electron flow has been shown to occur in vivo in cyanobacteria (Carpentier et al., 1984), in algae (Maxwell and Biggins, 1976; Ravenel et al., 1994), and in bundle sheath cells of C_4 plants (Herbert et al., 1990; Asada et al., 1993). In cyanobacteria, cyclic electron flow around PS I has been shown to provide extra ATP for different cellular processes, e.g. adaptation to salt stress conditions (Jeanjean et al., 1993). In the bundle sheath cell chloroplasts of C_4 plants, PS II is low or undetectable (Woo et al., 1970) and ATP supply is totally dependent upon PS I-mediated cyclic electron transport (Leegood et al., 1981).

In C_3 plants, PS I-driven cyclic electron flow has been studied mainly in vitro on isolated chloroplasts or thylakoids with addition of artificial cofactors or reduced ferredoxin (Bendall and Manasse, 1995). Under those conditions, the redox poise was proposed to play an important role in the regulation of the rate of cyclic electron flow (Arnon and Chain, 1975; Heber et al., 1978; Fork and Herbert, 1993), with neither full reduction of the chloroplast electron transport chain (Ziem-Hanck and Heber, 1980) nor excessive oxidation allowing cyclic electron flow to occur in vitro. In intact leaves, PS I-mediated cyclic electron flow in far-red light was analyzed indirectly by measuring the light-scattering signal at 535 nm, which reflects changes in the trans-thylakoid pH gradient (Heber et al., 1992, 1995; Cornic et al., 2000). Cyclic electron transport around PS I can also be estimated indirectly by measuring the re-reduction rate of the oxidized primary electron donor in PS I (P_{700}^+) after switching off the far-red light (Maxwell and Biggins, 1976; Asada et al., 1992). It was observed that this rate measured in leaves of C_3 plants (e.g. Burrows et al., 1998) was considerably much slower than that measured in the green alga *Chlamydomonas reinhardtii* (Maxwell and Biggins, 1976; Ravenel et al., 1994) or in

* Corresponding author; e-mail michel.havaux@cea.fr; fax 33–4–42256265.

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cyanobacteria (Mi et al., 1992), suggesting a very slow recycling of electrons around PS I in vivo. The existence of cyclic electron transport in vivo in C_3 plants has also been questioned by photoacoustic measurements in far-red light (Herbert et al., 1990), which allow a direct and quantitative measure of energy storage (ES) by cyclic electron flow around PS I (for review, see Malkin and Canaani, 1994). This method has confirmed the existence of cyclic electron transfer reactions in C_4 plants, algae, and cyanobacteria (Herbert et al., 1990), but failed to show significant cyclic activity in C_3 plant leaves (Herbert et al., 1990; Havaux et al., 1991; Malkin et al., 1992).

New biochemical and genetic data support, however, the idea that cyclic electron flow around PS I occurs in vivo in C_3 plants. The plastid genome of higher plants contains *ndh* genes encoding peptides homologous to subunits of the proton-pumping NADH:ubiquinone oxidoreductase, a component of the mitochondrial respiratory chain (Ohyama et al., 1986; Shinozaki et al., 1986), and an NADH-dehydrogenase complex (Ndh) has been purified from pea (*Pisum sativum*) and barley (*Hordeum vulgare*) thylakoid membranes (Sazanov et al., 1998; Quiles et al., 2000). Inactivation of some *ndh* genes using plastid transformation of tobacco (*Nicotiana tabacum* cv Petit Havana) demonstrated the existence of a functional Ndh complex and its involvement in the transient nonphotochemical reduction of the PQ pool after a light to dark transition (Burrows et al., 1998; Cournac et al., 1998; Shikanai et al., 1998). Based on the study of chlorophyll fluorescence kinetics and the effects of inhibitors such as antimycin on tobacco leaf discs of an Ndh-less tobacco mutant, it was recently suggested that the Ndh complex could be involved in a PS I cyclic electron pathway operating in vivo in C_3 plants (Joët et al., 2000).

The apparent discrepancy between those results and the absence of measurable cyclic activity in vivo remains to be elucidated. Cyclic PS I activity is usually measured under very special conditions (PS I excitation by far-red light, PS II inhibition by 3-[3,4-dichlorophenyl]-1,1-dimethylurea [DCMU]) in which linear electron flow is diminished or even abolished. We assumed that the adequate redox poise supposedly required for cyclic electron flow in vivo is not achieved under those experimental conditions. In the present study, we have used photoacoustic spectroscopy and kinetic spectrophotometry to monitor cyclic electron transport around PS I in C_3 plants. A rapid electron cycling around PS I was induced in vivo by increasing the reduction level of the stromal NADP pool and of the intersystem electron carriers using anaerobic conditions or respiration inhibitors. The high cyclic activity of PS I thus obtained was different in wild-type (WT) tobacco and in a mutant lacking the Ndh complex, demonstrating the involvement of the Ndh complex in cyclic PS I activity.

RESULTS

Cyclic Electron Transport around PS I in Different Organisms

PS I-mediated cyclic electron flow was monitored in vivo in *Synechocystis* sp., maize (*Zea mays*) and tobacco using the photoacoustic technique (Fig. 1A). This technique measures the conversion of light energy to heat in an absorbing sample and hence the storage of light energy as chemical energy (photochemical ES; see "materials and Methods"; Malkin and Canaani, 1994). Figure 1A shows a typical in vivo photothermal signal generated by *Synechocystis* sp. cells deposited on a nitrocellulose filter and irradiated with modulated far-red light. Addition of a strong nonmodulated far-red light to the modulated light beam saturates PS I photochemistry, causing a noticeable rise in the photoacoustic signal. Thus, the comparison of the actual and maximal heat-emission signals provides a measure of the amount of absorbed light energy that was stored in intermediates of the photochemical processes (Malkin and Canaani, 1994). ES measured in the cyanobacterium *Synechocystis* sp. PCC 6803 under such conditions was close to 18%. As far-red light is almost exclusively absorbed in PS I, the measured ES is specifically related to the PS I function, reflecting ES in photochemical

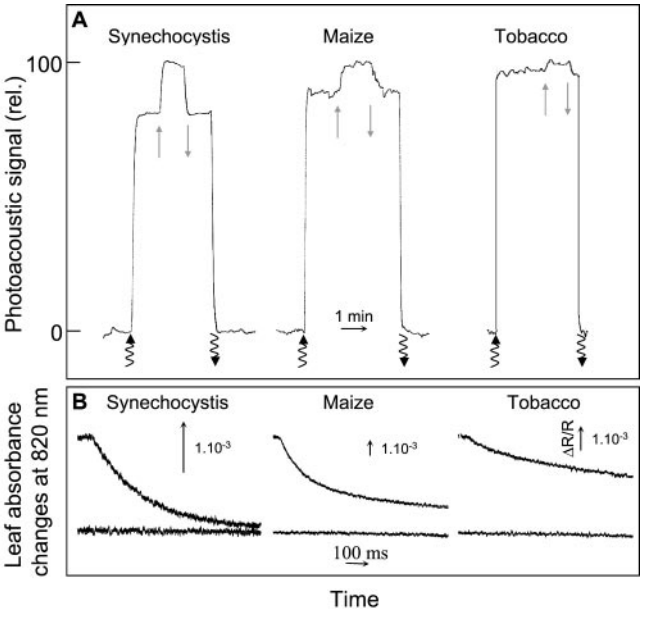

Figure 1. A, Photoacoustic signals generated by filter-deposited *Synechocystis* sp. cells and by water vacuum-infiltrated leaf discs of maize and tobacco, in wavelengths of measuring light absorbed predominantly by PS I (>715 nm; 10 Hz; 30 W m^{-2}). Upward-pointing arrows and downward-pointing arrows respectively indicate saturating far-red light (320 W m^{-2}) on and off. Thin wavy arrows represent the modulated measuring light. B, Dark re-reduction of P_{700}^+ after a far-red light period was monitored on the same samples and was expressed as Δ reflectance/reflectance (ΔR/R). Data expressing the half-time ($t_{1/2}$) of the dark P_{700}^+ reduction are means ± SD of six experiments.

products associated with the cycling of electrons around PS I (Canaani et al., 1989; Herbert et al., 1990). In higher plants, photoacoustic measurements must be conducted on water- or buffer-infiltrated leaves to eliminate the oxygen-evolution-related component of the photoacoustic signal (Malkin et al., 1992). In infiltrated leaves of maize (a C_4 plant), ES in far-red light (approximately 15%) was close to the activity measured in *Synechocystis* sp. In contrast, only a very small fraction (<5%) of the absorbed far-red light was used for photochemistry in infiltrated leaf discs of tobacco, a C_3 plant. This confirms a previous photoacoustic study of Herbert et al. (1990) who showed that C_3 plants exhibit no significant ES in far-red light.

Leaf absorbance measurements at 820 nm, which reflect changes in the redox state of the PS I reaction center (P_{700}), were also performed on the samples used for the photoacoustic measurements. P_{700} was oxidized by far-red light, and its subsequent reduction in the dark by stromal reductants was recorded (Fig. 1B). The half-time ($t_{1/2}$) of the dark re-reduction of P_{700}^+ after a period of far-red light was about 178 ± 12 ms in the cyanobacterium and 196 ± 29 ms in maize leaves. In tobacco, the re-reduction rate was considerably much slower, the $t_{1/2}$ value being close to 1,157 ± 113 ms. The latter value indicates a slow re-reduction of P_{700}^+, which can be attributed to a slow kinetics of electron donation from the stroma to the intersystem electron transport chain and/or a small pool of electron donors in the stroma after the far-red light illumination (Mi et al., 1992). In tobacco, it is possible that this pool is oxidized in far-red light and is regenerated very slowly in the dark.

Induction of Cyclic PS I Activity in C_3 Plants by Anaerobiosis

Assuming that the redox poise is a key factor in the induction of cyclic electron flow in C_3 plants, ES and the P_{700} dark reduction were measured under anaerobiosis, a condition known to reduce both the intersystem electron transport chain (Harris and Heber, 1993) and the stromal NADP pool (Joët et al., 1998). Anaerobiosis was reached in situ by placing tobacco leaf discs between two plastic wrap films impermeable to gas exchanges. Under those conditions, mitochondrial respiration consumes molecular O_2 in the gas phase of the leaf sample. This was checked by placing a leaf disc between a Clark-type O_2 electrode and a plastic film (data not shown). Oxygen was quickly consumed within 25 to 30 min, finally reaching the level obtained by flushing nitrogen in the chamber. We followed the changes in chlorophyll fluorescence emission (F_o and F_m levels) by tobacco leaf discs placed between two plastic films (Fig. 2). As previously reported by Harris and Heber (1993), obtention of anaerobiosis resulted in a noticeable increase in the dark level (F_o) of chlorophyll fluorescence (around +35%), indicating a partial reduction

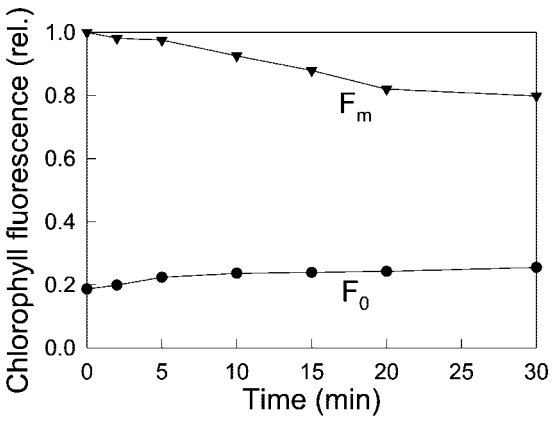

Figure 2. Changes in chlorophyll fluorescence of a film-enclosed leaf disc. The dark chlorophyll fluorescence F_o (●) was monitored by rapidly switching the nonactinic modulated measuring light on and off. The maximal fluorescence level F_m (▼) was determined by applying an 800-ms flash of saturating white light to the dark-adapted leaf discs.

of the PQ pool. At the same time, the maximal fluorescence level (F_m) progressively decreased. The latter effect probably reflects a transition from state 1 to state 2, which modified the light energy distribution between PS II and PS I in response to the reduction of the PQ pool via lateral movement of a fraction of the light-harvesting complexes II (Allen, 1992). This was confirmed by measuring 77 K chlorophyll fluorescence spectra of tobacco leaf discs in air or in anaerobiosis. The ratio between the F730 fluorescence peak (corresponding to PS I-associated pigments) and the F685 peak (PS II-associated pigments) was 3.49 ± 0.27 for leaf discs dark-adapted in air and 4.84 ± 0.19 for leaf discs placed between two plastic films, indicating that the latter leaves underwent a transition to state 2 in which light energy is redistributed in favor of PS I. However, we cannot exclude that part of the F_m quenching involves also other factors such as nonphotochemical fluorescence quenching related to a trans-thylakoidal ΔpH.

Photoacoustic measurements of ES in far-red light were conducted in tobacco leaves subjected to anaerobiosis. Figure 3A shows that anaerobiosis led to a considerable increase in ES by cyclic PS I activity from less than 5% to approximately 25%. The effect of anaerobiosis was also measured on the rate of P_{700} reduction after far-red light (Fig. 3B). In air, $t_{1/2}$ was about 1,157 ± 114 ms whereas anaerobic conditions drastically decreased the $t_{1/2}$ value to about 393 ± 89 ms, indicating a marked acceleration of the electron donation to P_{700}. The photoacoustic photothermal signal generated by tobacco leaf discs was measured at different fluence rates of far-red light, and Figure 4 shows the plot of ES versus the fluence rate. In air, ES was very low at any fluence rate of far-red light. In contrast, ES measured in anaerobiosis was much higher and significantly decreased with increasing far-red light fluence rate, indicating a progressive

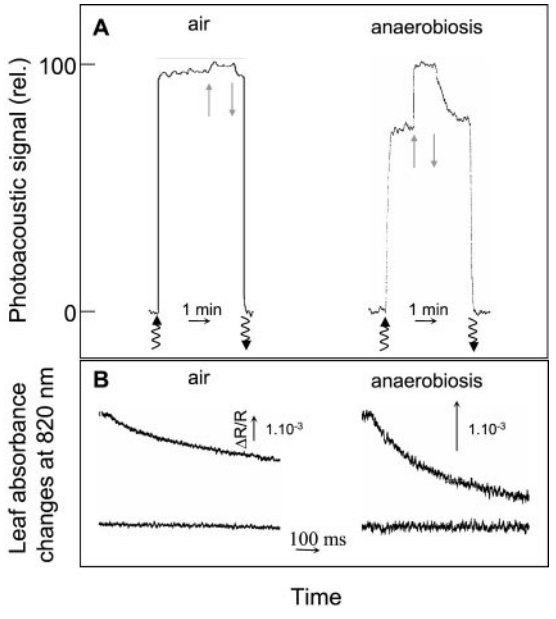

Figure 3. A, Photoacoustic signals (arbitrary units) generated by buffer vacuum-infiltrated (aerobiosis) or plastic film-enclosed (anaerobiosis) tobacco leaf discs were measured in wavelengths of measuring light absorbed predominantly by PS I (>715 nm; 10 Hz; 30 W m^{-2}). B, Dark re-reduction of P_{700}^+ after a far-red light period was measured on buffer vacuum-infiltrated (aerobiosis) and plastic film-enclosed (anaerobiosis) tobacco leaf discs. Data expressing the half-time ($t_{1/2}$) of the P_{700}^+ dark reduction are means ± SD of six experiments.

light saturation of cyclic PS I activity. The inset of Figure 4 represents the plot of ES^{-1} versus light fluence rate, which is linear (Havaux et al., 1989). The extrapolation of this plot to a fluence rate of zero gives an estimate of the maximal efficiency of photochemical ES. This maximal ES was close to 8% in aerobic conditions whereas it was increased to 30% to 50% in anaerobic conditions. ES measured in anaerobiosis was not inhibited by DCMU, thus confirming that ES measured in far-red light is specifically related to PS I photochemistry (Fig. 4).

Mechanisms by which anaerobiosis leads to a stimulation of PS I-mediated cyclic activity were then investigated. First, the stimulatory effect of anaerobiosis on the P_{700}^+ re-reduction rate was mimicked by inhibiting mitochondrial respiration either with myxothiazol or salicyl hydroxamic acid (SHAM; Table I). The use of two inhibitors is necessary to inhibit both the cytochrome respiratory pathway and the alternative oxidase pathway. Via chloroplast-mitochondria interactions, inhibition of respiration increased the reducing power in the chloroplasts leading to a rapid electron donation from the stroma to the intersystem chain, as did anaerobiosis. We observed the same phenomenon using antimycin A and SHAM (Table I), although antimycin A is also a potent inhibitor of the ferredoxin-dependent pathway of cyclic electron flow around PS I. This indicates that the latter pathway is not limiting for P_{700} re-reduction under our experimental conditions and it can be compensated by the other pathways of nonphotochemical reduction of intersystem electron carriers. It is interesting that P_{700}^+ re-reduction was also faster in leaves infiltrated with propyl gallate, a potent inhibitor of the newly discovered plastid terminal oxidase (Cournac et al., 2000). This protein has been recently overexpressed in tobacco leaves and was clearly shown to be involved in the dark oxidation of the PQ pool and to be sensitive to propyl gallate in planta (T. Joët, B. Genty, E.M. Josse, M. Kuntz, L. Cournac, and G. Peltier, unpublished data). This shows that electron flow to P_{700} can be enhanced by either increasing nonphotochemical reduction of PQ or decreasing its oxidation, suggesting that the reduction state of the PQ pool play a central role in the induction of cyclic electron flow.

Cyclic electron flow around PS I was searched in various C_3 plant species exposed to anaerobiosis. In air, ES was very low or even undetectable, whereas anaerobiosis caused a strong increase in ES in all species tested (Table II). As a consequence the phenomenon reported for tobacco is not restricted to this species but can be considered as a general response of C_3 plants to anaerobic conditions.

Anaerobiosis-Induced Cyclic Electron Transport around PS I in a Ndh-Less Tobacco Mutant

To further characterize PS I cyclic electron flow in C_3 plants, we performed photoacoustic and 820-nm absorbance measurements on leaves of a tobacco mutant lacking the plastid Ndh complex (Horvath et al., 2000). In Figure 5A, the reciprocal of ES measured in the WT and the mutant under anaerobic conditions was plotted versus the far-red light fluence rate. When photoacoustic measurements were performed

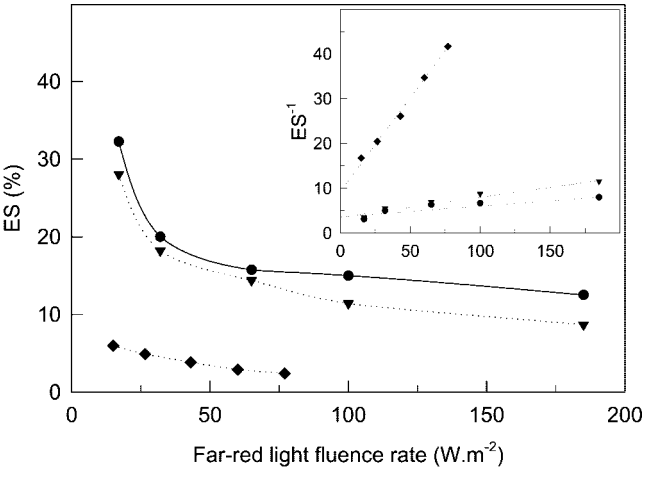

Figure 4. Plot of ES versus the fluence rate of the measuring far-red light (>715 nm, 10 Hz) for tobacco leaf discs under aerobic (◆) or anaerobic conditions (●). The effect of 50 μM DCMU under anaerobiosis is also shown (▼). Inset, Plot of the reciprocal of ES versus light fluence rate.

Table I. *Effects of various chemicals on the half-time ($t_{1/2}$) of the dark reduction of P_{700}^+ after far-red illumination (6.5 W m^{-2}; >715 nm)*

The measurements were performed in air, unless specified otherwise. Data are means ± SD of six experiments.

Treatment	P_{700} Re-Reduction
	$t_{1/2}$, ms
Control	1,157 ± 114
DCMU (20 μM)	1,135 ± 148
Antimycin A (5 μM)	1,118 ± 78
Myxothiazol (5 μM)	1,253 ± 233
SHAM (0.8 mM)	1,100 ± 117
Myxothiazol (5 μM) + SHAM (0.8 mM)	397 ± 95
Antimycin A (5 μM) + SHAM (0.8 mM)	440 ± 128
Propyl gallate (1 mM)	387 ± 85
Anaerobiosis	393 ± 89

using far-red light modulated at a low frequency of 10 Hz, no significant difference in ES was found between the WT and the Ndh-less mutant. This shows that ES by PS I cyclic electron flow can occur in the absence of the Ndh complex, via alternative electron transfer pathways. No significant difference was detected between WT and Ndh-deficient mutant on the basis of the half-time of the P_{700} re-reduction under anaerobiosis (Fig. 5B). However, increasing the modulation frequency to 22 Hz revealed a noticeable difference between the two genotypes (Fig. 5A). The linear plot of the mutant had a much steeper slope than the plot of the WT, indicating that PS I-driven cyclic electron transport was more rapidly saturated with increasing far-red light intensity in the Ndh-deficient mutant. The frequency dependence of ES in far-red light was analyzed in more detail in Figure 6. In WT leaves placed in anaerobiosis, a progressive decrease in ES was observed with increasing frequency of the modulated light. A similar frequency dependence of ES by cyclic PS I activity was previously observed in *C. reinhardtii* (Canaani et al., 1989). ES depends on the modulation frequency because this parameter reflects energy stored in photochemical products that decay with a time constant larger than the modulation frequency of excitation (Malkin and Canaani, 1994). Thus, at very low frequency, ES reflects long-lived intermediates. More

Table II. *Photoacoustically measured energy storage (%) by PS I cyclic activity in leaves of various C_3 plants illuminated with far-red light (30 W·m^{-2}; 10 Hz) in air and in anaerobiosis*

nd, Not detected.

C_3 Plant Species	Air	Anaerobiosis
B. napus	6	42
Barley	5.5	28
Arabidopsis	nd	17
Potato	nd	37
P. koreana × *P. trichocarpa* Torr. & Gray	9	41
A. pseudoplatanus	8	18

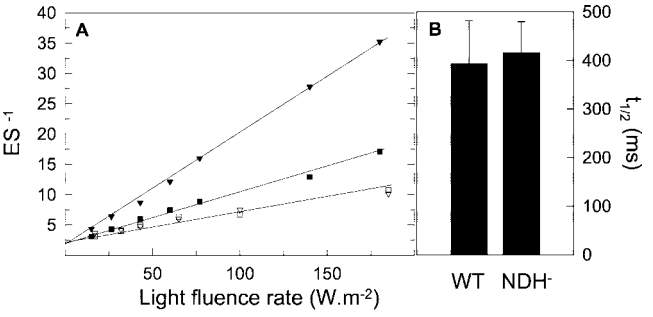

Figure 5. A, Plot of the reciprocal of ES versus the fluence rate of the measuring far-red light (>715 nm) under anaerobic conditions. ES was measured in WT (□, ■) and Ndh-less mutant (▽, ▼) at 10 Hz (□, ▽) and 22 Hz (■, ▼). B, Dark re-reduction of P_{700}^+ after a far-red light period was measured on WT and Ndh-less mutant tobacco leaf discs under anaerobiosis. Data expressing the half-time ($t_{1/2}$) of the P_{700}^+ dark reduction are means ± SD of 12 experiments.

precisely, at a given frequency of modulation f, ES corresponds to photochemical products of electron transport that persist for $1/2\pi f$ seconds after excitation (Canaani et al., 1989). Considering a maximal ES value of about 20% (at modulation frequencies close to zero), we estimated that the frequency corresponding to the half-value of the maximal ES was around 26 Hz in the WT. From this value, an apparent reaction half-time of 6 ms can be calculated for the limiting step of the reaction responsible for the measured ES. It is striking that ES measured in the Ndh-deficient mutant decreased much more rapidly with the modulation frequency, and the half-value of the maximal ES value was reached at around 12 Hz. In this case, the photochemical reaction responsible for ES in far-red light has a half-time of 13 ms. As expected, ES was low at any frequency in both WT and Ndh-less mutant leaves in air. The data of Figure 6 show that cyclic electron transfers around PS I in WT

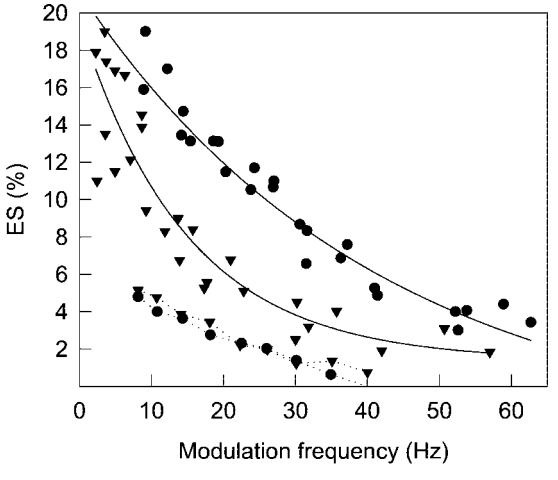

Figure 6. Plot of ES versus the frequency of the modulated light for WT (●) and Ndh-less mutant leaves (▼). ES was measured under aerobic (dotted lines) or anaerobic conditions (full lines) using far-red modulated light (>715 nm; 40 W m^{-2}). The experimental points are the results of three independent experiments.

tobacco and in the Ndh-deficient mutant are kinetically different, the electron cycle being much slower in the latter one.

DISCUSSION

Rapid Cyclic Electron Flow around PS I in Vivo in C_3 Plants

In this study, cyclic electron flow around PS I was triggered in vivo by placing tobacco leaf discs in anaerobiosis. The maximal ES level measured in tobacco leaves in far-red light was equivalent to that measured in cyanobacteria and C_4 plants. The frequency dependence of ES measured in anaerobiosis showed that the frequency corresponding to one-half of the maximal ES was about 26 Hz, indicating that the rate constant of the photochemical reaction responsible for the ES was about 6 ms. This is to compare to the 2 ms previously observed in *C. reinhardtii* (Canaani et al., 1989). To the best of our knowledge, this is the first time that such a high and rapid cyclic electron flow around PS I is measured in vivo in C_3 plants. Moreover, anaerobiosis-induced cyclic PS I activity was observed in several C_3 plant species, indicating that this phenomenon is a general feature of C_3 plants (Table II). The half-time of P_{700}^+ re-reduction measured in tobacco leaf discs was also accelerated in anaerobiosis from 1,200 ms (in air) to 400 ms which is very close to the $t_{1/2}$ reported for isolated thylakoids under anaerobiosis (Scheller, 1996). This value corresponds to a rapid electron donation to P_{700} which is of the same magnitude of that measured in *Synechocystis* sp. and maize (respectively close to 180 and 200 ms) or that reported for *C. reinhardtii* (220 ms; Ravenel et al., 1994) and other unicellular algae (150 ms; Maxwell and Biggins, 1976). Our results are in accordance with previous studies performed by Heber et al. (1978) who observed a significant increase in the light scattering of spinach leaves illuminated with far-red light by flushing with nitrogen. Since this signal is indirectly related to the thylakoid pH gradient, these authors proposed that PS I-mediated cyclic activity was stimulated by anaerobiosis.

Involvement of the Ndh Complex in Cyclic Electron Flow

The rapid cyclic electron transport around PS I in anaerobiosis is related to the Ndh complex activity. The cyclic PS I activity in Ndh-less mutant was kinetically different from that of the WT as shown by the more abrupt decrease of ES with increasing frequency. This indicates a slower cycling of electrons in the absence of functional Ndh complex. The photochemical reaction responsible for the measured ES in far-red light had a calculated half-time of approximately 13 ms, which is much higher than the value measured in the WT (approximately 6 ms). It is interesting that PS I-mediated cyclic electron flow was not inhibited in Ndh-less mutant leaves of tobacco at very low frequencies of modulated light (<10 Hz) indicating the existence of cyclic electron pathways independent of the Ndh complex. Those alternative pathways could be the antimycin-sensitive pathway (Cleland and Bendall, 1992; Joët et al., 2000) or a pathway involving alternative NAD(P) H dehydrogenases (Corneille et al., 1998; Cournac et al., 1998). The rapid disappearance of ES with increasing frequency in the Ndh-less mutant shows that these additional pathways are slow and that the predominant pathway in the WT is dependent on the Ndh complex. It should incidentally be noted that the photoacoustic method revealed differences in kinetics of cyclic electron flow whereas the P_{700}^+ re-reduction rate measured in the dark was equivalent in the WT and the Ndh-less mutant. This can be explained by the fact that the photoacoustic technique measures the energy stored during the complete electron cycle in far-red light whereas the P_{700} reduction rate in the dark reflects electron donation to P_{700} from stromal donors. This rate depends not only on the kinetics of electron transfer from stromal donors to the intersystem chain but also on the size of the stromal electron donor pool. Thus, the $t_{1/2}$ value is expected to depend on the experimental conditions (Mi et al., 1992). It has been shown in cyanobacteria that $t_{1/2}$ measured after continuous far-red light represents the donation from respiratory donors, and depletion of respiratory donors in dark-starved cells resulted in a very slow reduction of P_{700}^+ (Mi et al., 1992). Then, $t_{1/2}$ cannot be taken as an absolute value of the turnover time of P_{700} during cyclic electron transport around PS I. Rather, it should be considered as an indicator of the potential capacity of electron donation to P_{700} from stroma electron donors. In tobacco, re-reduction of oxidized P_{700} was possibly determined mainly by the re-generation of stromal electron donors after switching off the far-red light. This phenomenon was probably more limiting for the P_{700} reduction rate than the absence of Ndh, thus explaining why the WT and the Ndh-deficient mutant could not be distinguished on the basis of the P_{700} reduction kinetics. In contrast, in the photoacoustic experiments, the frequency dependence of ES allows the kinetics of the electron cycle to be analyzed and provides information on the lifetime of the intermediates that limit the energy-storage reaction (Malkin and Canaani, 1994; Malkin, 1996). Also, it cannot be excluded that some phenomena needed for cyclic electron flow are deactivated in the dark and are thus not observable in the P_{700} redox change experiments. Further studies are probably required to confirm our interpretations.

The involvement of the Ndh complex in cyclic electron transport around PS I is thus demonstrated in this study, confirming a number of previous reports that have hypothesized the role of the Ndh complex

in cyclic electron transport via measurements of nonphotochemical reduction of the PQ pool in the dark (Burrows et al., 1998; Shikanai et al., 1998; Cournac et al., 1998). It was recently shown that photosynthesis of the Ndh-less mutant leaves was highly sensitive to antimycin A, and it was concluded to the participation of the Ndh complex in cyclic electron flow (Joët et al., 2000). Cyclic electron flow via the NADPH pool has already been described in the cyanobacterium *Synechocystis* sp., where lesions in the *ndh* genes were observed to cause a strong slowdown of the P_{700} turnover in far-red light (Mi et al., 1992, 1995) and a marked inhibition of ES in far-red light (Jeanjean et al., 1998). At last, an NAD(P) H dehydrogenase activity involved in PS I cyclic activity has also been suggested in *C. reinhardtii* where several independent pathways may coexist in vivo (Ravenel et al., 1994). It should be pointed out that the involvement of Ndh in cyclic electron transport around PS I can be direct by allowing rapid recycling of electrons from NADPH to the PQ pool or indirect as a redox poise regulator (compare with below).

The Redox Poise Controls Cyclic PS I Activity in C_3 Plants

It is likely that PS I-mediated cyclic electron transport is controlled by the atmospheric O_2 concentration in C_3 plants via changes in the redox state of intersystem electron carriers and/or stromal reductants. The PQ pool was found to be partially reduced in tobacco leaves exposed to anaerobiosis in plastic wrap films. In a previous study, the NADPH to NADP ratio, as measured in vivo by the blue-green fluorescence emission, was observed to increase noticeably in anaerobiosis (Joët et al., 1998). A control of PS I-driven cyclic electron transfer by the reduction state of intersystem electron carriers and through the $NADP^+$ to NADPH ratio was previously suggested from in vitro data obtained on isolated C_3 chloroplasts (Arnon and Chain, 1975, 1979; Slovacek et al., 1980; Takahama et al., 1981; Hosler and Yocum, 1985, 1987) and from light scattering measurements on leaves (Heber et al., 1978). In those experiments, it was shown that cyclic activity is modulated by varying the O_2 partial pressure to modify the redox poise of the intersystem electron carriers (Arnon and Chain, 1975, 1979; Scheller, 1996). This suggests that adequate redox poise was not achieved in leaves placed in air and illuminated with far-red light, thus explaining why PS I-mediated cyclic activity was not detectable in vivo in C_3 plants.

Adequate redox poise was also induced in air either by inhibiting mitochondrial oxidases using myxothiazol or antimycin and SHAM or by inhibiting a chloroplastic PQ oxidation pathway using propyl gallate. In the former case, the cytosolic NAD(P) H, which cannot be oxidized by mitorespiration, is rerouted toward chloroplasts because of the existence of redox interactions between mitochondria and plastid (for review, see Hoefnagel et al., 1998), hence stimulating nonphotochemical reduction of the intersystem electron carriers (Gans and Rebeillé, 1990). In leaves treated with propyl gallate, inhibition of a plastid oxidase involved in nonphotochemical oxidation of the PQ pool, led to an over-reduction of the intersystem electron carriers. Those mechanisms, by which the PQ pool is nonphotochemically reduced and subsequently reoxidized in the dark using molecular oxygen as a terminal acceptor, are commonly described as chlororespiration (Bennoun, 1982; Peltier et al., 1987; Nixon, 2000). From our data, we conclude that the entire chlororespiratory electron transfer chain, i.e. the nonphotochemical reduction as well as the nonphotochemical oxidation of the PQ pool, may control the redox poise of intersystem electron transport chain in vivo in C_3 plants, which in turn controls PS I-mediated cyclic electron flow.

Anaerobic conditions were used in this study as an experimental trick that allows measurement of cyclic electron transport in far-red light. Under physiological conditions, the adequate redox state of the PQ pool may be achieved by the PS II activity, especially when PS I acceptors are not fully available. Under our experimental conditions, however, cyclic electron flow is measured in far-red light, which cannot stimulate PS II activity, and cyclic electron flow is then artificially activated in anaerobiosis by simultaneous stimulation of nonphotochemical PQ reduction and inhibition of PQ oxidation by the plastid oxidase. The situation described here in higher plant chloroplasts is close to that occurring in cyanobacteria where respiration and photosynthesis electron transfer chains share the PQ pool in common (Scherer, 1990). It is interesting that Schubert et al. (1995) reported a stimulation of PS I-driven cyclic electron flow, estimated by the photochemical ES in far-red light and by the P_{700}^+ dark reduction in the cyanobacterium *Fremyella diplosiphon* when treated with KCN, a well-known inhibitor of cytochrome c oxidase.

Transition to the light state 2, which is activated when the intersystem redox carriers are reduced (Allen, 1992), took place in tobacco leaves exposed to anaerobiosis. During transition to state 2, the cytochrome b_6/f complex was shown to accumulate in the stroma lamellae of maize and *C. reinhardtii* (Vallon et al., 1991) where both PS I and the Ndh complex are located (Horvath et al., 2000; Sazanov et al., 1998). This could possibly facilitate PS I cyclic activity. One may then suppose that reduction of the PQ pool can indirectly favor cyclic electron flow via state-transition-related migration of cyt b_6f to the vicinity of PS I. It is interesting that inhibitors of mitochondrial respiration were also reported to induce reduction of the PQ pool and transition to state 2 in *C. reinhardtii* (Bulté et al., 1990).

One intriguing question is why the adequate redox poise for cyclic electron transport requires specific conditions in C_3 plants and is naturally observed in

other types of plants under far-red light conditions. Under normal conditions, it is possible that nonphotochemical reduction of the PQ pool is not sufficient to achieve the adequate redox poise of intersystem electron carriers. Cyclic electron flow will be triggered by a reduction of the PQ pool mediated either by an imbalance in chlororespiration activity between nonphotochemical reduction and oxidation of PQs or by PS II activity. This could be the case for example during induction of photosynthesis or in high light, where the intersystem electron carriers are partly reduced. The Calvin cycle uses more ATP than NADPH so that a high photosynthetic rate will lead to an increased NADPH to ATP ratio (Osmond, 1981). It has been suggested that one function of cyclic electron transfer around PS I is to synthesize extra ATP to adjust the NADPH to ATP ratio (Bendall and Manasse, 1995). Our observation that PS I cyclic activity is controlled by the redox poise of the chloroplasts is consistent with this function: An accumulation of NADPH will trigger the cyclic electron flow, thus compensating the ATP deficit.

MATERIALS AND METHODS

Plant Material and Preparation of Leaf Samples

WT tobacco (*Nicotiana tabacum* cv Petit Havana) and *ndhB*-inactivated mutant (see Horvath et al., 2000) were grown on compost in a phytotron (25°C day/20°C night) under a photon flux density of 350 μmol photons m^{-2} s^{-1} (photoperiod, 12 h) supplied by quartz halogen lamps (HQI-T 400W/DV, Osram, Germany). Plants were watered with one-half-strength Hoagland nutritive solution. Leaf samples were taken from plants aged 5 to 8 weeks. Potato (*Solanum tuberosum* L. cv Desirée), Arabidopsis cv Colombia, barley (*Hordeum vulgare* L. cv Plaisant), and *Brassica napus* cv Orphée were cultivated under the same conditions, whereas poplar (*Populus koreana* × *Populus trichocarpa Torr. & Gray* cv Peace) was grown in a greenhouse. Samples from *Acer pseudoplatanus* were harvested outside.

Anaerobiosis was induced by placing leaf discs (12 mm in diameter) between two plastic films of barrier food wraps (Saran, Dow Chemical, Midland, MI) and keeping them in the dark for 60 min before photoacoustic or absorbance measurements. Because of respiration, O_2 was rapidly depleted in the leaf discs, as controlled with an O_2 electrode at 25°C.

Treatments with Inhibitors

DCMU treatment was performed on whole leaves that were infiltrated with 50 μM DCMU via their petiole through the transpiration flux for 5 h. PS II inhibition by DCMU was checked by chlorophyll fluorescence yield measurements with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). For other treatments, tobacco leaf discs were stripped by removal of lower epidermis and soaked for 90 min in petri dishes containing distilled water and various inhibitors. The inhibitors were added diluted in methanol (maximal final methanol concentration was 0.5%). Control leaf discs were soaked in petri dishes containing water and 0.5% (v/v) methanol.

Photoacoustic Measurements of Photochemical ES

ES by cyclic electron flow around PS I was measured in vivo using the photoacoustic technique (Herbert et al., 1990; Havaux et al., 1991; Ravenel et al., 1994). The photoacoustic spectrometer is described in Ravenel et al. (1994). Leaf discs, placed in the photoacoustic chamber, were illuminated with modulated far-red light (>715 nm). The far-red light fluence rate was measured with a LI-COR radiometer (Li-185B/Li-200SB, LI-COR, Lincoln, NE). PS I photochemistry was saturated with a strong background far-red light (>715 nm, 320 W m^{-2}). ES was calculated from the amplitude of the maximal photothermal photoacoustic signal (Apt^+ measured when the strong far-red light was added to the modulated measuring light) and the actual photothermal signal amplitude (Apt):

$$ES = (Apt^+ - Apt)/Apt^+ \quad (1)$$

The plastic wrap film is impermeable to O_2 and, therefore, abolishes the gas-exchange-related photoacoustic signal that can appear at low modulation frequencies. When the plastic film was not used, leaf discs were soaked for 3 h in an osmoticum buffer (25 mM phosphate buffer, pH 7.0, 200 mM sorbitol, 10 mM KCl, and 2 mM $MgCl_2$) to eliminate the photobaric component of the photoacoustic signal as described by Malkin et al. (1992). *Synechocystis* sp. cells were filtered under pressure through an MF-Millipore filter (cellulose nitrate/acetate, SS type, 3-μm pore size, Millipore, Bedford, MA). The cells deposited on the filter (diameter, 1.2 cm) were then placed in the photoacoustic cell for measurement.

Redox State of P700

Changes in the redox state of the reaction center P_{700} of PS I were monitored via leaf absorbance changes at around 820 nm (Schreiber et al., 1988). A Walz PAM-101 system connected to an ED-800-T emitter/detector unit (Walz) was used in the reflection mode (Schreiber et al., 1988). The rate of P_{700} re-reduction was measured with a storage oscilloscope (Tektronix 5111A, Tektronix, Guernsey, Channel Islands) after a period of far-red light (6.5 W m^{-2}, >715 nm).

Room Temperature and Low Temperature Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence spectra were recorded in liquid nitrogen (77 K) with leaf discs dark-adapted for 30 min using a bifurcated light guide connected to a Perkin-Elmer LS50B spectrofluorometer (Perkin-Elmer, Beaconsfield, UK). The wavelength of the excitation light beam was 440 nm.

Room temperature chlorophyll fluorescence was measured with a PAM-2000 chlorophyll fluorometer (Walz). F_o was excited with a dim red light modulated at 600 Hz. Fm was induced by a 800-ms pulse of intense white light.

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Chapitre III

Implication de la protéine PTOX dans l'oxydation non photochimique du pool de PQ

Si le complexe Ndh est impliqué dans la réduction non photochimique du pool de PQ, l'existence d'une chaîne chlororespiratoire implique qu'une oxydase terminale intervienne dans l'oxydation non photochimique du pool de PQ. A ce titre, nous nous sommes interressés à PTOX, une protéine chloroplastique homologue aux oxydases alternatives mitochondriales, dont il a été récemment montré qu'elle était impliquée dans la biosynthèse des caroténoïdes (Carol et al., 1999 ; Wu et al., 1999).

L'étude de mutants de *Chlamydomonas reinhardtii* dépourvus de PS I a permis de montrer l'implication de PTOX dans l'oxydation du pool de PQ par l'oxygène moléculaire (voir article joint Cournac et al. (2000) Phil Trans R Soc London B 355 : 1447-1454). Nous avons ensuite cherché à caractériser la fonction de PTOX au sein des chloroplastes des végétaux supérieurs. Pour ce faire, nous avons entrepris de surexprimer la protéine PTOX d'*Arabidopsis thaliana* dans le tabac (voir article joint Joët et al., soumis). La caractérisation de plantes transgéniques surexprimant PTOX, par des mesures de fluorescence de la chlorophylle et d'échange d'oxygène, nous a permis de montrer que PTOX est impliquée dans l'oxydation non photochimique du pool de PQ et utilise l'oxygène moléculaire comme accepteur terminal d'électrons. A l'obscurité le complexe Ndh et PTOX sont connectés au pool de PQ formant les deux éléments d'une chaîne de transfert d'électrons chlororespiratoire. L'effet du propyl gallate sur les plantes sauvages a permis de montrer un rôle similaire pour la protéine PTOX native au sein des chloroplastes de tabac.

A. Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration

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Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration

Laurent Cournac[1*], Eve-Marie Josse[2], Thierry Joët[1], Dominique Rumeau[1], Kevin Redding[3], Marcel Kuntz[2] and Gilles Peltier[1]

[1]*CEA/Cadarache, DSV, DEVM, Laboratoire d'Ecophysiologie de la Photosynthèse, 13108 Saint-Paul-lez-Durance, France*

[2]*Laboratoire de Génétique Moléculaire des Plantes, CNRS-Université Joseph Fourier, UMR 5575, BP 53X, 38041 Grenoble Cedex 09, France*

[3]*Department of Chemistry and Coalition for Biomolecular Products, The University of Alabama, Tuscaloosa, AL 35487-0336, USA*

Besides electron transfer reactions involved in the 'Z' scheme of photosynthesis, alternative electron transfer pathways have been characterized in chloroplasts. These include cyclic electron flow around photosystem I (PS I) or a respiratory chain called chlororespiration. Recent work has supplied new information concerning the molecular nature of the electron carriers involved in the non-photochemical reduction of the plastoquinone (PQ) pool. However, until now little is known concerning the nature of the electron carriers involved in PQ oxidation. By using mass spectrometric measurement of oxygen exchange performed in the presence of ^{18}O-enriched O_2 and *Chlamydomonas* mutants deficient in PS I, we show that electrons can be directed to a quinol oxidase sensitive to propyl gallate but insensitive to salicyl hydroxamic acid. This oxidase has immunological and pharmacological similarities with a plastid protein involved in carotenoid biosynthesis.

Keywords: chlororespiration; quinol oxidase; chloroplast; oxygen; *Chlamydomonas*

1. INTRODUCTION

During photosynthesis, two photosystems (PS II and PS I), coupled through an electron transfer chain, transform light energy to chemical energy. Besides this main electron transport pathway, called the 'Z' scheme of photosynthesis, alternative pathways such as cyclic electron transport around PS I (Arnon 1955; Heber & Walker 1992; Ravenel *et al*. 1994) and a respiratory chain called chlororespiration (Bennoun 1982; Peltier *et al*. 1987) have been identified in thylakoid membranes. Recent work has supplied some clues on the molecular properties of electron carriers involved in alternative pathways. First, a NAD(P)H dehydrogenase complex (Ndh), encoded by plastidial *ndh* genes, has been characterized in thylakoid membranes (Guedeney *et al*. 1996; Sazanov *et al*. 1998). Inactivation of *ndh* genes by plastid transformation was simultaneously performed by different laboratories (Burrows *et al*. 1998; Shikanai *et al*. 1998; Kofer *et al*. 1998; Cournac *et al*. 1998). It was shown that the Ndh complex is involved in the non-photochemical reduction of plastoquinones (PQ) occurring in the dark after a period of illumination and it was further suggested that this complex is involved in cyclic electron flow around PS I and in chlororespiration. Although not characterized at a molecular level, the existence of other activities, such as ferredoxin quinone reductase activity (Bendall & Manasse 1995; Endo *et al*. 1998) or non-electrogenic NAD(P)H dehydrogenase activity—different from the Ndh complex and involved in PQ reduction (Corneille *et al*. 1998)—have been reported in thylakoids.

If the nature of electron carriers involved in non-photochemical reduction of the PQ pool appears better understood, the nature of electron carriers involved in plastoquinol oxidation remains a subject of controversy. Recently, a homologue to mitochondrial alternative oxidase has been simultaneously characterized in *Arabidopsis* thylakoid membranes by two different laboratories (Carol *et al*. 1999; Wu *et al*. 1999). This enzyme, which is encoded by the nuclear gene *immutans*, has been shown to be essential during carotenoid biosynthesis and it was assumed that it might catalyse plastoquinol oxidation and be involved in chlororespiration. In contrast, based on experiments performed *in vitro*, Casano *et al*. (2000) recently proposed a chlororespiration model in which plastoquinol oxidation would be achieved by a plastidial peroxidase, H_2O_2 being used as an electron acceptor.

In order to elucidate the nature of the chlororespiratory oxidase, we have used photosynthetic mutants of the green alga *Chlamydomonas* and performed mass spectrometric measurements. Mass spectrometry, using ^{18}O-labelled O_2,

*Author for correspondence (laurent.cournac@cea.fr).

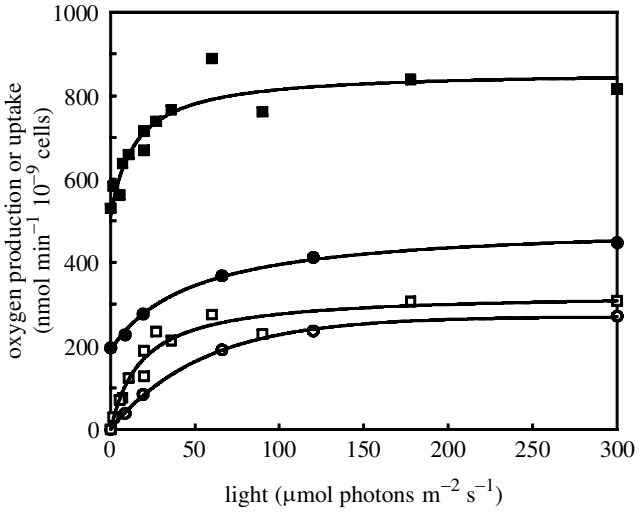

Figure 1. Photosynthetic oxygen evolution measured in intact *Chlamydomonas* cells. Measurements were performed in two independent PS-I-deficient strains showing different chlorophyll contents. Circles, mutant strain *psaAΔ*1, 0.44 mg chlorophyll 10^{-9} cells. Squares, mutant strain *psaBΔ*7, 2.3 mg chlorophyll 10^{-9} cells. Rates of PS II oxygen production (deduced from $^{16}O_2$ enrichment of the medium) and of oxygen uptake (deduced from $^{18}O_2$ depletion of the medium) are plotted versus the illumination intensity. Open circles, *psaAΔ*1 production; closed circles, *psaAΔ*1 uptake; open squares, *psaBΔ*7 production; closed squares, *psaBΔ*7 uptake.

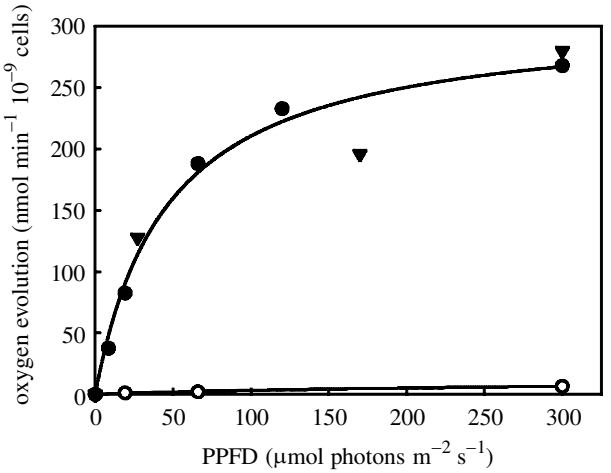

Figure 2. Effect of DCMU and loss of the cyt b_6f complex on the PS-II-driven O_2 production measured in intact *Chlamydomonas* cells using $^{18}O_2$ to label dissolved oxygen and monitoring $^{16}O_2$ (produced by PS II) and $^{18}O_2$ (taken up) with mass spectrometry. Closed circles, mutant strain *psaAΔ* deficient in PS I; open circle, mutant strain *psaAΔ* in the presence of 10 μM DCMU; closed triangles, double mutant *psaAΔ petAΔ* deficient in PS I and in the cyt b_6f complex.

is a powerful way to determine whether electrons produced at PS II (measured as unlabelled O_2 from water photolysis) are diverted towards O_2 or to another electron acceptor. By performing such measurements in *Chlamydomonas* preparations lacking either the PS I complex or the cytochrome (cyt) b_6f complex, we show that electrons provided by PS II can be diverted at a significant rate towards a chloroplast quinol oxidase. Based on the similarity of immunological (Cournac *et al*. 2000) and pharmacological properties between the *immutans* encoded plastid terminal oxidase (PTOX) in *Arabidopsis* and the plastoquinol oxidizing activity in *Chlamydomonas*, we propose the involvement of a quinol oxidase in chlororespiration.

2. EXPERIMENTAL PROCEDURES

Chlamydomonas reinhardtii cells were grown on a tris–acetate–phosphate medium (TAP). Algal cultures were maintained at 25 °C under continuous agitation and low illumination (about 1 μmol photons m^{-2} s^{-1}). The wild-type strain used in this work was isolated as a *mt*$^+$ segregant of a cross between two strains isogenic to the 137c strain (Harris 1989). The original deletions of *psaA* and *psaB* (chloroplast genes which encode essential subunits of PS I) were made in this strain as previously reported (Fischer *et al*. 1996). Marker recycling allowed subsequent transformations to delete the chloroplast *petA* gene, which encodes for an essential subunit of cyt b_6f (Cournac *et al*. 2000).

Prior to thylakoid isolation, the cells were harvested, centrifuged (600 *g*, 5 min) and washed once with 15 mM HEPES-KOH, pH 7.2. After centrifugation in the washing medium (600 *g*, 5 min), the pellet (around 5×10^8 cells) was resuspended in 10 ml buffer A (0.3 M sorbitol, 50 mM HEPES-KOH, pH 7.8, 2 mM EDTA, 5 mM $MgCl_2$) supplemented with 1% bovine serum albumin (BSA). Thylakoids were obtained through disruption in a French press chamber of the cells at 5000 psi in buffer A + 1% BSA (two runs). After disruption, broken or intact cells and heavy parts were discarded by centrifugation (600 *g*, 3 min). The supernatant was then centrifuged at 3000 *g*. The pellet (thylakoid fraction) was resuspended in 300–500 μl buffer A (without BSA) and stored on ice until used in the experiments. Oxygen exchange assays were conducted in buffer A without BSA.

Thylakoid membranes were resuspended in buffer A up to 1.5 ml in the measuring chamber. For measuring O_2 exchange on whole cells, algal cultures were harvested in exponential growth phase, centrifuged, washed and resuspended in buffer A. One and a half millilitres of the suspension was placed in the measuring chamber: a Clarke electrode-type thermostated and stirred cylindrical vessel (Hansatech, Norfolk, UK) fitted onto a mass spectrometer connecting device. Dissolved gases were directly introduced in the ion source of the mass spectrometer (model MM 14-80, VG instruments, Cheshire, UK) through a Teflon membrane as described in Cournac *et al*. (1993). For O_2 exchange measurements, the sample was sparged with N_2 to remove $^{16}O_2$, and $^{18}O_2$ (95% ^{18}O isotope content, Euriso-Top, Les Ulys, France) was then introduced to achieve an O_2 concentration in solution close to that in equilibrium with normal air. Light was supplied by a fibre-optic illuminator (Schott, Main, Germany) and neutral filters were used to vary light intensity. Unless specified, experiments shown here were performed at 300 μmol photons m^{-2} s^{-1} incident light. All gas exchange measurements were performed at 25 °C. The chloroplastic extracts were used as quickly as possible after extraction.

The portion of the *Arabidopsis immutans* cDNA coding for the entire mature peptide (PTOX) was PCR-amplified and inserted in the *Escherichia coli* expression vector pQE31 (Qiagen, Courtaboeuf, France) as described elsewhere (Cournac *et al*. 2000; Josse *et al*. 2000). The recombinant membrane protein PTOX which possesses a 6 His-tag was

Table 1. *Effect of electron acceptors on oxygen exchange*

(Measured in intact cells or thylakoids of the *psa AΔ* and *psaAΔ petAΔ* strains. *E*, photosynthetic O_2 evolution; δU, light-induced oxygen uptake (uptake in the light − uptake in the dark).)

	psaAΔ				*psaAΔ petAΔ*			
	nmol O_2 min^{-1} mg^{-1} chlorophyll				nmol O_2 min^{-1} mg^{-1} chlorophyll			
	intact cells		thylakoids		intact cells		thylakoids	
treatment	*E*	δU	*E*	δU	*E*	δU	*E*	δU
control	520	560	120	130	570	510	140	170
FeCN	620	640	670	80	550	590	170	160
DCBQ	980	10	900	0	1100	30	930	10

produced in *E. coli*. After induction, cells were lysed and membranes were recovered upon centrifugation at 100 000 *g* for 1 h. Pelleted membranes were resuspended in tris–HCl 0.2 M, pH 7.5, sucrose 0.75 M. Oxygen consumption was measured in a Clark O_2 electrode chamber (Hansatech). A typical assay contained 100 g membrane protein in the following buffer: tris–maleate 50 mM, pH 7.5, KCl 10 mM, $MgCl_2$ 5 mM, EDTA 1 mM, decyl-plastoquinone 0.2 mM.

3. RESULTS

PS-I-deficient algae obtained by inactivation of *psaA* or *psaB* genes were illuminated in the presence of ^{18}O-labelled O_2, and O_2 exchange was determined by mass spectrometry by following concentration changes in $^{18}O_2$ and $^{16}O_2$. As previously reported in nuclear mutants deficient in PS I (Peltier & Thibault 1988) or in plastid mutants (Cournac *et al*. 1997), significant O_2 evolution by PS II was measured, this phenomenon being accompanied by a simultaneous stimulation of O_2 uptake (figure 1). In these conditions, no change in the apparent respiration rate was observed, since light-dependent O_2 production and light-stimulated O_2 uptake are of the same amplitude. Light-dependent oxygen evolution was measured in different PS-I-deficient mutants. The maximal (light-saturated) activity was variable when expressed on a chlorophyll basis (from 120–600 nmol O_2 min^{-1} mg^{-1} chlorophyll), but was more constant when normalized to the cell number (250–350 nmol O_2 min^{-1} 10^{-9} cells) or to the protein amounts (8–13 nmol O_2 min^{-1} mg^{-1} protein), probably reflecting differences in chlorophyll contents between strains. Figure 1 shows O_2 exchange data in two strains with different chlorophyll contents. Comparable rates of maximal electron transfer activity were reached by both strains, but strains with higher chlorophyll contents were found to be more efficient at low light intensities. Note that the maximum rate of O_2 evolution in PS-I-deficient mutants represented about 10% of the maximal O_2 production rate measured in wild-type cells (not shown). The PS-II-dependent O_2 production was previously reported to be strongly affected by inhibition of mitochondrial respiration (Peltier & Thibault 1988; Cournac *et al*. 2000). However, we found that the light-driven activity of PS II was unaffected by the increase in respiration consecutive to acetate addition (data not shown) or by the level of basal respiration observed in different mutant strains (see figure 1). In contrast, the PS-II-dependent activity was found to vary during the algal cell cycle. Maximal activity was present during exponential growth, but severely decreased during the stationary phase (data not shown).

DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor blocking photosynthetic electron transfer between Q_A (the primary quinone acceptor of PS II) and Q_B (the secondary quinone acceptor, which exchanges with the PQ pool), strongly inhibited the PS-II-driven O_2 evolution (figure 2). Also, the PS-II-dependent electron flow was observed in the absence of the cyt b_6f complex in a *Chlamydomonas* double mutant *psaAΔ petAΔ* lacking both PS I and cyt b_6f (figure 2). Similar results were obtained in the single mutant (*petAΔ*) deficient in cyt b_6f or in the presence of 1 μM dibromothymoquinone (DBMIB), a cyt b_6f inhibitor (not shown). We conclude from these data that the PQ pool, but not the cyt b_6f complex, is involved in the PS-II-dependent pathway.

In order to determine the maximal PS II activity present in thylakoids of PS-I-deficient mutants, we measured photosynthetic O_2 evolution in the presence of artificial electron acceptors like 1,5-dichlorobenzoquinone (DCBQ) or potassium ferricyanide (FeCN) (table 1). In the presence of DCBQ, PS II activity was increased, indicating that PS II was not limiting the electron transport activity. In parallel, the light stimulation of O_2 uptake was completely suppressed. A similar effect was observed in whole cells and in a double mutant lacking PS I and the cyt b_6f complex (table 1). An increase in O_2 evolution was also observed in thylakoids of PS-I-deficient mutants when using FeCN as an electron acceptor. This effect was accompanied by a *ca*. 40% diminution of the light-induced stimulation of O_2 uptake (table 1). However, FeCN had no significant effect on the PS-II-dependent O_2 evolution in intact cells, which is explained by the fact that this compound cannot enter intact cells. Interestingly, FeCN has no significant effect on O_2 exchange rates measured in thylakoids from the *Chlamydomonas* strain lacking both PS I and the cyt b_6f complex (*psaAΔ petAΔ*, table 1).

A gene (*immutans*) encoding a plastid protein (PTOX) showing a high homology with the mitochondrial alternative oxidase, was recently discovered in *Arabidopsis thaliana* (Carol *et al*. 1999; Wu *et al*. 1999). As it was not easy to assay oxidase activity in *Arabidopsis* chloroplasts,

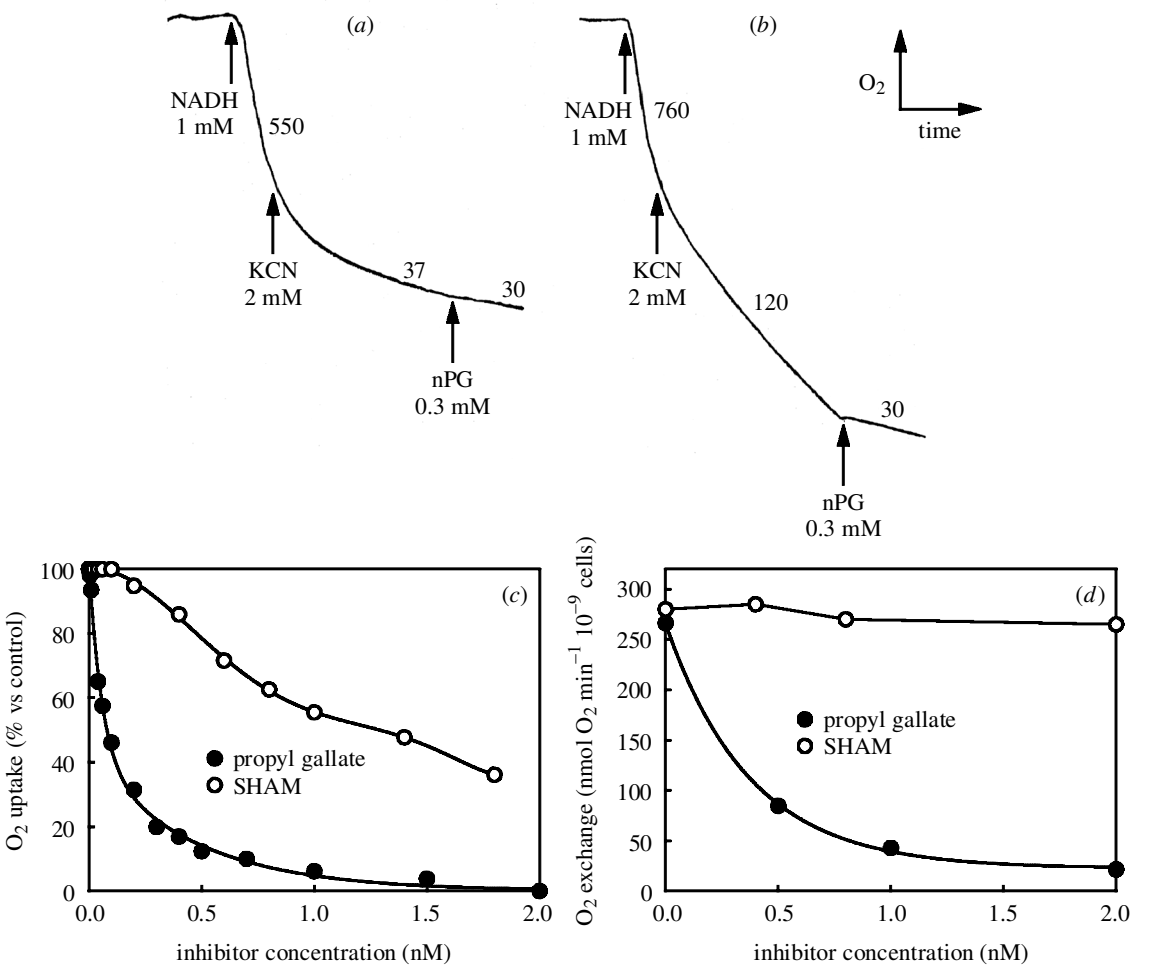

Figure 3. (*a*,*b*) Effects of sequential addition of KCN and propyl gallate on O_2 uptake induced by addition of NADH in membranes of *E. coli* from (*a*) control cells and (*b*) cells expressing the PTOX protein. O_2 uptake are given in nmol min^{-1} mg $protein^{-1}$. (*c*) Sensitivity of the PTOX-induced O_2 uptake to propyl gallate and SHAM in membranes of *E. coli*. (*d*) Sensitivity of PS-II-driven O_2 exchange to propyl gallate and SHAM in *Chlamydomonas* mutants deficient in PS I.

due to the low abundance of PTOX in chloroplasts and to the possible occurrence of mitochondrial cross-contamination, PTOX was produced as a recombinant protein in *E. coli*. After induction of the chimeric gene, the oxidase activity of membrane preparations was assayed by adding NADH and measuring oxygen consumption. KCN (1 mM) was used to inhibit oxygen consumption due to the cytochrome oxidase pathway (Josse *et al.* 2000). Expression of PTOX in *E. coli* membranes conferred a significantly higher cyanide-resistant oxygen consumption (figure 3*a*,*b*). Propyl gallate and salicylhydroxamic acid (SHAM) are well-known inhibitors of the mitochondrial alternative oxidase. The PTOX-dependent and cyanide-resistant oxidase activity was sensitive to propyl gallate (figure 3*a*–*c*), but at least ten times less sensitive to SHAM (figure 3*c*). The PS-II-dependent activity of PS-I-inactivated mutants showed comparable sensitivity to propyl gallate and was insentive to SHAM up to 2 mM (figure 3*d*).

4. DISCUSSION

(a) *Characteristics of photosynthetic electron transport in PS-I-deficient mutants*

In agreement with previous findings (Peltier & Thibault 1988; Cournac *et al.* 1997; Redding *et al.* 1999), results shown in this paper show that significant electron transport activity occurs from PS II to O_2 in PS-I-deficient *Chlamydomonas* mutants. Based on the effect of DCMU and on measurements performed in strains lacking the cyt b_6f complex, we conclude that the electron flow between PS II and molecular O_2 involves the thylakoid PQ pool, but not the cyt b_6f complex. Due to its electronic requirements and to its insensitivity to relative oxygen species (ROS) scavengers, PQ oxidation has been concluded to involve an enzymatic process reducing molecular O_2 into water (Cournac *et al.* 2000). As demonstrated here using an artificial electron acceptor for PS II (DCBQ), the activity of oxidase limits PS-II-dependent O_2 evolution in the absence of PS I. This explains why the maximal rates of O_2 evolution in PS-I-deficient cells are five to 20 times lower than that in wild-type cells, where PS I and cyt b_6f cooperate to reoxidize the PQ pool. However, light saturation curves of PS II activity indicate that PS-II-driven electron transport is limited by chlorophyll content at low light, and by oxidase content at high light. This suggests that oxygen uptake is not directly dependent on chlorophyll and is not related to chlorophyll photo-oxidation, further supporting the involvement of an enzymatic process in plastoquinol oxidation.

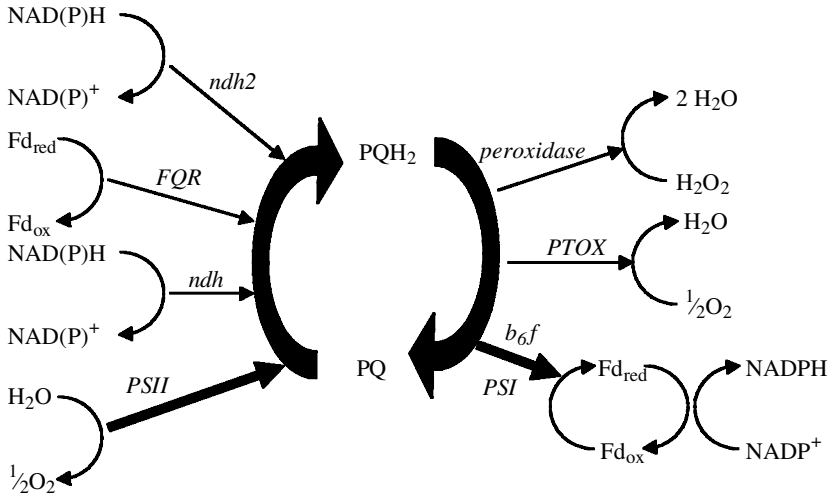

Figure 4. Schematic representations of the different plastoquinone (PQ) reduction and plastoquinol (PQH_2) oxidation pathways now evidenced in thylakoid membranes. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; ndh, complex I-like NAD(P)H dehydrogenase; ndh2, alternative NADH dehydrogenase; FQR, ferredoxin–quinone reductase; PTOX, plastid terminal oxidase (the quinol oxidase described in this paper).

Interestingly, we found that FeCN could accept electrons from PS II in PS-I-deficient strains containing cyt b_6f, but not in cyt b_6f-depleted strains. This shows that FeCN can interact with the intersystem photosynthetic electron transport chain, probably at the level of cyt *f* as previouly reported (Wood & Bendall 1976). This also indicates that the cyt b_6f complex of PS-I-deficient mutants keeps the ability to oxidize plastoquinol and to compete efficiently with the quinol oxidase.

The influence of various inhibitors has given us clues as to the nature of the chloroplast oxidase involved in this plastoquinol oxidation. Cyanide, which has been reported to impair chlororespiration (Buchel & Garab 1995; Bennoun 1982; Peltier *et al.* 1987) or cyanobacterial quinol oxidases (Howitt & Vermaas 1998; Buchel *et al.* 1998), had no effect unless very high concentrations were used (Cournac *et al.* 2000). The absence of effect of FeCN on plastoquinol oxidation in the cyt b_6f-deleted strain (table 1) also precludes the involvement of a soluble transporter such as soluble cytochromes, since FeCN can interact with such cytochromes, as shown in mitochondria (Hoefnagel *et al.* 1995).

(b) *Similarities between the* Chlamydomonas *plastoquinol oxidase and PTOX*

In plant mitochondria, quinol oxidation can be accomplished either by the cyt bc_1 complex (cyanide-sensitive pathway), or directly to molecular O_2 through an alternative oxidase (cyanide-insensitive pathway). Alternative oxidases have been reported to be inhibited by compounds such as SHAM or propyl gallate (Siedow 1980). We found that propyl gallate, but not SHAM, inhibited the PS II-to-O_2 electron flow in *C. reinhardtii* mutants deficient in PS I. Interestingly, Berthold (1998) reported the existence of different mutant forms of the *Arabidopsis thaliana* mitochondrial alternative oxidase that are resistant to SHAM but remain sensitive to propyl gallate, thus showing that sensitivity to these two inhibitors is separable.

Recently, two laboratories simultaneously reported the existence, in *Arabidopsis thaliana*, of a gene (*immutans*) coding for a plastid protein (PTOX) showing homology with mitochondrial alternative oxidases (Carol *et al.* 1999; Wu *et al.* 1999). Based on the phenotype of mutants affected in the *immutans* gene, it was concluded that PTOX is involved in carotenoid biosynthesis, more particularly in phytoene desaturation. The authors proposed a model in which PTOX would catalyse reoxidation of plastoquinol to PQ, using O_2 as a terminal acceptor. We have shown that PTOX, when expressed in *E. coli*, confers a KCN-insensitive quinol oxidase activity. In this assay, the plastid oxidase PTOX is sensitive to propyl gallate and much less sensitive to SHAM. Interestingly, PTOX appears to be more resistant to both inhibitors than mitochondrial alternative oxidase (Berthold 1998). Figure 3 indicates that both PTOX and the *Chlamydomonas* plastoquinol oxidase have similar sensitivities towards propyl gallate. Both activities show resistance towards SHAM, but PTOX appears significantly more sensitive. Differences in SHAM sensitivity can be explained by different hypotheses.

(i) The oxidases are not exactly the same, and the *Chlamydomonas* type is more resistant to SHAM.
(ii) The O_2 uptake in *E. coli* membranes is more sensitive to SHAM than in thylakoids, some modifications of its properties being induced by the expression system (a chimeric gene in a bacterial context).

Based on similar effects of inhibitors on PTOX and PS-II-driven electron flow, we conclude that the enzyme responsible for plastoquinol oxidation in *Chlamydomonas* is closely related to PTOX. This conclusion is further supported by immunological data (Cournac *et al.* 2000).

(c) *Oxygen, reactive oxygen species and chlororespiration*

We have concluded from our experiments that the major part of chloroplast O_2 uptake is due to the activity of a quinol oxidase that uses molecular O_2 as an electron acceptor and is sensitive to propyl gallate but insensitive to cyanide. Such a sensitivity to inhibitors appears contradictory to the involvement in chlororespiration of a cyanide-sensitive oxidase, as concluded by different authors (Bennoun 1982; Peltier *et al.* 1987; Buchel & Garab 1995). On the other hand, the use of molecular O_2 as a terminal acceptor is not consistent with the model of chlororespiration recently proposed by Casano *et al.* (2000). Indeed, based on experiments performed on an

in vitro reconstructed system, these authors proposed that plastoquinol oxidation was achieved by a plastid peroxidase using H_2O_2 as a terminal acceptor.

We cannot exclude at this stage the existence of different pathways of non-photochemical oxidation of plastoquinols, one involving a quinol oxidase and the other a peroxidase. According to Casano *et al.* (2000), the participation of a peroxidase might explain the cyanide sensitivity through an inhibition of superoxide dismutase. These different pathways might be differentially regulated depending on the environmental conditions. One might expect that the peroxidase pathway, provided that its existence is confirmed *in vivo*, would be associated with conditions generating ROS such as stress or senescence. On the other hand, PTOX would be involved in reactions occurring during the early biogenesis of chloroplasts (see Carol *et al.* 1999). This would be consistent with the higher plastoquinol oxidation activity observed during active phases of division. In this respect, it would be interesting to determine whether the peroxidase pathway is triggered during phases of senescence or in stress conditions.

It seems now likely that just as the non-photochemical PQ reduction pathways are diverse, so too are the chloroplastic O_2 (or ROS) uptake pathways (figure 4). Unravelling the molecular basis of these activities and their physiological significance will be an exciting task for the future.

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Discussion

J. Barber (*Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK*). Have you estimated the stoichiometric level of the PQ oxidase and the NAD complex in normal chloroplasts relative to the major complexes such as PS I, PS II and cyt b_6f?

G. Peltier. Sazanov *et al.* (1996) have estimated that the Ndh complex of pea chloroplasts represented less than 0.2% of total thylakoid membrane proteins (about one complex every 100 photosynthetic chains). It is therefore clearly a minor component of thylakoid membranes compared with the major complexes such as PS I, PS II or cyt b_6f. We have not yet estimated the amounts of the chlororespiratory oxidase present in thylakoid membranes. However, one may speculate that, like the Ndh complex, it represents a minor component of thylakoid membranes. This probably explains why these enzymes have not been discovered earlier.

K. Niyogi (*Department of Plant and Microbial Biology, University of California, Berkeley, USA*). Have you found any evidence for reverse electron flow through the Ndh complex?

G. Peltier. No, we have no evidence for this occurrence of reverse electron flow through the Ndh complex. Initially, the inhibition of the PS-II-dependent O_2 evolution by respiratory inhibitors observed in intact *Chlamydomonas* cells from PS-I-deficient mutants was interpreted by the generation of NAD(P)H through an energy-dependent reverse electron transfer occurring through a putative chloroplast Ndh complex and a transfer of reducing equivalents from the chloroplast to the mitochondria (Peltier & Thibault 1988). However, as shown here, and as recently published by Cournac *et al.* (2000), PS-II-dependent O_2 evolution could be measured in chloroplasts from PS-I-deficient mutants and was insensitive to respiratory inhibitors. We have concluded from these data that the PS-II-dependent O_2 evolution observed in PS-I-deficient mutants is due to a diversion of electrons towards a chloroplast oxidase. The inhibition of the PS-II-dependent electron flow by respiratory inhibitors would be explained by a competition between PS II and stromal donors for the reduction of the PQ pool. Moreover, it now seems clear that the plastid genome of most unicellular algae lacks *ndh* genes. In *Chlamydomonas*, non-photochemical reduction of the PQ pool is probably achieved by a non-electrogenic enzyme (for a review, see Cournac *et al.* 2000). This argues against the existence of a reverse electron flow, which would be only possible with an electrogenic complex. In higher plants, such a possibility cannot be excluded, since the Ndh complex is probably electrogenic, but no evidence for such a mechanism has been obtained until now.

C. H. Foyer (*Department of Biochemistry and Physiology, IACR-Rothamsted, UK*). The role of the alternative oxidase in the mitochondrial electron transport chains is considered to be prevention of over-reduction of the PQ pool and hence uncontrolled electron drainage to oxygen. Would you consider that a possible role of the chloroplast oxidase is to prevent over-reduction of the PQ pool and hence photoinhibition?

G. Peltier. Such a role should be considered. It is clear from our experiments that in PS-I-deficient *Chlamydomonas* mutants electrons can be diverted towards the chloroplast oxidase. Whether this reaction occurs *in vivo* in the presence of active PS I remains to be answered. One may speculate that in conditions where PS I is partially inhibited, for instance during introduction of photosynthesis, where electron acceptors are lacking, or during low temperature photoinhibition, diversion towards the oxidase may prevent over-reduction of the PQ pool.

A. Laisk (*Department of Plant Physiology, Tartu University, Estonia*). Is chloroplast Ndh a proton translocating enzyme? The background of my question is that with G. Edwards we measured quantum yields of C_4 plants and found them to be 15% higher than possible considering the known efficiency of cyclic electron transport. The discrepancy could be resolved with the assumption that proton-translocating Ndh participates in the cyclic electron flow in C_4-plant bundle-sheath chloroplasts.

G. Peltier. Based on the homology between plastid Ndh genes and bacterial genes encoding subunits of the NADH dehydrogenase complex, it seems likely that the chloroplast Ndh complex involved in chlororespiration and cyclic electron flow around PS I is a proton-translocating enzyme. In C_4 plants, Kubicki *et al.* (1996) have reported strong expression of Ndh genes in bundle-sheath chloroplasts. Possibly, the participation of such a proton-translocating complex to cyclic electron flow around PS I may explain increases in quantum yields.

H. C. P. Matthijs (*Department of Microbiology, University of Amersterdam, The Netherlands*). Professor Badger asked about the role of Ndh 1 in PS I cyclic, and pointed to the fact that Ndh 1, in addition to a role in PS I cyclic,

may be directly linked to CO_2 uptake. To this I added that in a Ndh-1-less mutant of the cyanobacterium *Synechocystis* which cannot grow in low CO_2 condition, growth on low CO_2 can be restored after (NaCl) stress. In this stress, PS I cyclic activity increases two- to three-fold, flavodoxin and FNR induction up to 20–30 times. This shows an intimate relationship between PS I cyclic and CO_2 uptake (Jeanjean *et al.* 1998).

G. Peltier. Our recent studies on Ndh-inactivated mutants (Horvath *et al.* 2000), have shown a role of the Ndh complex during photosynthesis under low CO_2 concentration, for instance during a stomatal closure induced by water limitation. Our interpretation is that under such conditions the requirement of photosynthetic CO_2 fixation for ATP is higher. To fix one CO_2, an ATP–NADPH ratio of 1.5 is needed under non-photorespiratory conditions, but under photorespiratory conditions this ratio increases up to 1.65. We proposed that cyclic electron flow around PS I mediated by the Ndh complex is a putative CO_2 concentrating mechanism similar to that occurring in cyanobacteria or algae. In this respect, the existence in the chloroplast genome of an open reading frame encoding a protein sharing homologies with a cyanobacterial and *Chlamydomonas* protein involved in CO_2 concentrating mechanisms is rather intriguing. However, until now, such a mechanism has not been evidenced in higher plant chloroplasts.

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B. Involvement of a plastid terminal oxidase in both chlororespiratory and photosynthetic electron transport chains: evidence from overexpression in tobacco

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Involvement of a Plastid Terminal Oxidase in Plastoquinone Oxidation as Evidenced by Expression of the *Arabidopsis thaliana* Enzyme in Tobacco*

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Thierry Joët‡§, Bernard Genty‡, Eve-Marie Josse¶, Marcel Kuntz¶, Laurent Cournac‡, and Gilles Peltier‡‖

From the ‡CEA Cadarache, Direction des Sciences du Vivant, Département d'Ecophysiologie Végétale et de Microbiologie, Laboratoire d'Ecophysiologie de la Photosynthèse, Unité Mixte de Recherche 163 CNRS CEA, Univ-Mediterranée CEA1000, 13108 Saint-Paul-lez-Durance, Cedex and ¶Laboratoire de Génétique Moléculaire des Plantes, Université Joseph Fourier and CNRS (Unité Mixte de Recherche 5575), BP53, 38041 Grenoble Cedex 9, France

Chlororespiration has been defined as a respiratory electron transport chain in interaction with photosynthetic electron transport involving both non-photochemical reduction and oxidation of plastoquinones. Different enzymatic activities, including a plastid-encoded NADH dehydrogenase complex, have been reported to be involved in the non-photochemical reduction of plastoquinones. However, the enzyme responsible for plasquinol oxidation has not yet been clearly identified. In order to determine whether the newly discovered plastid oxidase (PTOX) involved in carotenoid biosynthesis acts as a plastoquinol oxidase in higher plant chloroplasts, the *Arabidopsis thaliana PTOX* gene (*At-PTOX*) was expressed in tobacco under the control of a strong constitutive promoter. We showed that At-PTOX is functional in tobacco chloroplasts and strongly accelerates the non-photochemical reoxidation of plastoquinols; this effect was inhibited by propyl gallate, a known inhibitor of PTOX. During the dark to light induction phase of photosynthesis at low irradiances, At-PTOX drives significant electron flow to O_2, thus avoiding over-reduction of plastoquinones, when photosynthetic CO_2 assimilation was not fully induced. We proposed that PTOX, by modulating the redox state of intersystem electron carriers, may participate in the regulation of cyclic electron flow around photosystem I.

In photosynthetic organisms like photosynthetic bacteria or cyanobacteria, photosynthesis and respiration operate in close interaction within the same membranes where they share some electron transport components such as the plastoquinone (PQ)[1] pool (1). In chloroplasts, the existence of a respiratory electron transport chain (chlororespiration) in interaction with photosynthesis has been suggested (2, 3), and this activity has been proposed to originate from the cyanobacterial ancestor of chloroplasts (1). Chlororespiration would involve non-photochemical reduction of the PQ pool and subsequent oxidation by a plastoquinol terminal oxidase. Non-photochemical reduction of PQs is a well established phenomenon that likely occurs during cyclic electron transfer reactions around photosystem I (PS I). A plastid-encoded NADH dehydrogenase (Ndh) complex showing homologies with bacterial complex I has been characterized in thylakoid membranes from higher plants (4–7). Inactivation of some *ndh* genes using plastid transformation of tobacco showed the involvement of the Ndh complex in non-photochemical reduction of PQs (8, 9). It was proposed that the Ndh complex participates in both chlororespiration and cyclic electron transfer around PS I (8–11). A role of the Ndh complex in cyclic electron flow around PS I was recently confirmed by photoacoustic measurements performed in tobacco *ndh* mutants (12). It should be noted that alternate activities, such as a putative ferredoxin-PQ reductase (FQR) (13, 14) or a Ndh-2 type activity may also be involved in these processes (15, 16).

Nevertheless, the involvement of a plastid terminal oxidase in chlororespiration has been the subject of controversy during the last decade (2, 3, 16, 17). Initially, the existence of chlororespiration was based mainly on the effect of respiratory inhibitors such as cyanide and CO on the redox state of the PQ pool (2). However, such effects can be alternatively explained by an inhibition of mitochondrial respiration and the existence of redox interactions between chloroplasts and mitochondria (16–20). Recently, the study of an *Arabidopsis* mutant (*immutans*) showing a variegated phenotype led to the identification of a protein involved in carotenoid biosynthesis (21, 22). Based on sequence homology with mitochondrial alternative oxidases, this protein was suggested to act as a plastid terminal oxidase (PTOX) (21, 23). Expression of *Arabidopsis* PTOX (At-PTOX) in *Escherichia coli* conferred a cyanide-resistant O_2 uptake sensitive to propyl gallate, a known inhibitor of alternative oxidases (24, 25). In PS I-less mutants of the green algae *Chlamydomonas reinhardtii*, a limited but significant electron flow from photosystem II (PS II) to molecular O_2 was measured. Based on the effects of inhibitors (insensitivity to KCN and CO and sensitivity to propyl gallate) on this process and on the detection of a thylakoid protein that cross-reacted with an antibody raised against PTOX, it was proposed that the chlororespiratory O_2 uptake is because of a *Chlamydomonas* homologue of PTOX (24). In higher plants, an involvement of PTOX in PQ oxidation has not been experimentally evidenced. Based on the study of a reconstituted system, Casano *et al.* (6) proposed that a peroxidase using hydrogen peroxide as an electron acceptor may be involved in chlororespiration.

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§ Present address: Dept. of Infectious Diseases, St. George's Hospital Medical School, Cramer Terrace, SW 17 ORE London, UK.

‖ To whom correspondence should be addressed. Tel.: 33-4-42257651; Fax: 33-4-42256265; E-mail: gilles.peltier@cea.fr.

[1] The abbreviations used are: PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Ndh, NADH dehydrogenase complex; PS I and II, photosystem I and II, respectively PTOX, plastid terminal oxidase; WT, wild type; MES, 2-(*N*-morpholino)ethanesulfonic acid; At-PTOX, *A. thaliana* PTOX; RT, reverse transcriptase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

To get further insight into the function of PTOX and in particular to determine whether this protein can achieve quinol oxidation in chloroplasts, tobacco plants constitutively expressing At-PTOX have been generated. We show that At-PTOX facilitates the oxidation of reduced PQs using O_2 as a terminal acceptor.

EXPERIMENTAL PROCEDURES

Plant Material—Tobacco plants (*Nicotiana tabacum* var. *petit havana*) were grown on compost in a phytotron (25 °C day/20 °C night; 12-h photoperiod) under an irradiance of 300 μmol photons·m^{-2}·s^{-1} supplied by quartz halogen lamps (HQI-T 400W/DV, Osram, Germany). Plants were watered with a half-diluted Hoagland's nutritive solution.

Production of Transgenic At-PTOX Tobacco Plants Overexpressing At-PTOX—The *Arabidopsis thaliana* PTOX cDNA (GenBank™ accession number AJ004881) was used as a template for PCR amplification using the primers 5′-CCGCTCGAGCCTGACGGAGATGGCGGCGATTTCAGG-3′ and 5′-CCCGAGCTCTTATTAACTTGTAATGGATTTCTTGAGGC-3′, respectively, containing an *Xho*I and an *Sac*I restriction site at the 5′ and 3′ end. The amplified fragment started 9 bp upstream to the coding sequence of the At-PTOX cDNA and contained two stop codons (the start codon and two stop codons are underlined). After digestion, the amplified fragment was introduced in a sense orientation into a plant binary vector (pKYLX71). Expression of At-PTOX was driven by a double sequence of the cauliflower mosaic virus ^{35}S-labeled constitutive promoter (26). The recombinant plasmid was introduced by electroporation into *Agrobacterium tumefaciens* (strain C58), which was used for tobacco transformation employing the standard leaf disc transformation method (27). Two independent transformation experiments were carried out, and six transformants were recovered on a kanamycin-selective medium (100 mg·liter^{-1}). Two independent transgenic lines ($PTOX_1^+$ and $PTOX_2^+$), overexpressing high amounts of PTOX, were selected and were self-pollinated. The T1 generation was used for further experiments.

Preparation of Osmotically Lysed Chloroplasts for O_2 Exchange and Chlorophyll Fluorescence Measurements—Leaves were harvested at the end of the night period, and intact chloroplasts were isolated at 4 °C on a Percoll gradient according to a modification of the method described by Mills and Joy (51). Approximately 30 g of leaves were ground in a blender for 2 s in 100 ml of medium A containing 330 mM sorbitol, 50 mM Tricine-NaOH, pH 7.8, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM ascorbic acid, and 5 mM dithiothreitol. After filtration through 250- and 60-μm nylon net, followed by centrifugation (2000 × *g*, 3 min), the crude extract was resuspended in medium A (dithiothreitol-free) and layered onto a Percoll step gradient formed with two layers of medium A containing 90 and 40% (v/v) Percoll, respectively. After centrifugation in a swing out rotor at 3,500 × *g* for 15 min, intact chloroplasts were recovered from the 40:90% Percoll interphase, washed with 60 ml of medium A, pelleted at 2,000 × *g* for 3 min, and osmotically lysed by resuspension in 10 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride for 30 min. Lysed chloroplasts were diluted at a final concentration of 200 μg of chlorophyll·ml^{-1} in 30 mM Hepes-KOH buffer, pH 7.5, containing 0.3 M sorbitol, 5 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $NaHPO_4$, 50% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride. Aliquots of the chloroplast preparation were stored at −20 °C. For O_2 exchange and chlorophyll fluorescence measurements, aliquots were resuspended in 30 mM Hepes-KOH buffer, pH 7.5, containing 0.3 M sorbitol, 5 mM NaCl, and 10 mM $MgCl_2$. $(F_m - F_0)/F_m$ measured on chloroplasts samples was 0.7 (±0.02, 6 experiments). Anaerobiosis was achieved by addition of glucose (20 mM) and glucose oxidase (2 mg·ml^{-1}) to the chloroplast suspension. Reactive oxygen species generated by the glucose oxidase activity were scavenged by adding superoxide dismutase (500 units·ml^{-1}) and catalase (1,000 units·ml^{-1}).

Mass Spectrometric O_2 Exchange Measurements—For mass spectrometric measurements of O_2 exchange, osmotically lysed chloroplasts (20 μg of chlorophyll·ml^{-1}) were placed in the measuring chamber (1.5-ml reaction volume). The sample was sparged with N_2 to remove $^{16}O_2$, and $^{18}O_2$ (95% ^{18}O isotope content, Euriso-Top, Les Ulys, France) was then introduced to reach an O_2 concentration close to the equilibrium with air. Dissolved gases were introduced into the ion source of the mass spectrometer (model MM 14–80, VG Instruments, Cheshire, UK) through a Teflon membrane. Light was supplied by a fiber optic illuminator (Schott, Main, Germany) supplying a light intensity of 150 μmol photons·m^{-2}·s^{-1}. All gas exchange measurements were performed at 25 °C. The use of $^{18}O_2$ allowed the *in vivo* determination of O_2 evolution by PS II (originating from the photolysis of water which is not enriched) in the presence of O_2 consuming processes.

Chlorophyll Fluorescence Measurements in Chloroplasts and Leaves—Chlorophyll fluorescence was measured at 25 °C using pulse-modulated fluorimeters (PAM 101–103 and PAM 2000, Walz, Effeltrich, Germany for chloroplasts and leaves, respectively). The maximal chlorophyll fluorescence level (F_m) was measured under a 0.8–1-s saturating pulse (about 8,000–10,000 μmol photons·m^{-2}·s^{-1}) in dark-adapted leaves, on which the basal fluorescence (F_0) was recorded before the pulse. The maximal photochemical yield of PS II was determined as $(F_m - F_0)/F_m$. Fluorescence levels F_m, F_s (fluorescence in the light), $F_{m'}$ (maximal fluorescence in the light, using a saturating pulse), and $F_{0'}$ (basal fluorescence of light-adapted leaves, recorded after rapid reoxidation of the PQ pool using far-red light) were used to calculate PS II photochemical yield ($F_{m'} - F_s/F_{m'}$), non-photochemical quenching ($qN = 1 - F_{m'}/F_m$), and photochemical quenching ($qP = F_{m'} - F_s/F_{m'} - F_{0'}$) under different irradiances (28). Apparent photosynthetic electron transport rates (μmol electrons·m^{-2}.s^{-1}) were estimated as $(F_{m'} - F_s)/F_{m'} \times PFFD_i \times LA \times 0.5$, where $PFFD_i$ is the incident photosynthetic photon flux density; LA is the leaf absorbance (0.84), and 0.5 the factor accounting for the light partition between the two photosystems.

For chlorophyll fluorescence measurements in stripped leaf discs, leaf samples were placed on a wet paper filter at 25 °C in ambient air. Chlorophyll fluorescence measurements on attached leaves were performed using the gas exchange cuvette of a Licor gas exchange system (LI-6400, Li-Cor Inc, Lincoln, NE) to control leaf temperature (25 °C) and gas atmosphere. Illumination was provided by a homemade red (663 nm) LEDs source.

Inhibitor Treatment of Leaf Discs—Leaf discs were sampled from 5- to 8 week-old plants. After stripping the lower epidermis, leaf discs were soaked in water for 60 min. 2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB, 50 μM final concentration) or propyl gallate (1 mM final concentration) was added diluted in methanol (maximal final methanol concentration was 0.5%). Control leaf discs were soaked in water containing methanol. It should be noted that the DBMIB concentration (50 μM) used for leaf discs was much higher than the concentration generally used to obtain specific inhibition of the cytochrome b_6/f complex on isolated thylakoids (1 μM). Despite this relatively high concentration necessary to obtain an effect in leaf discs, we checked that DBMIB did not act as an electron acceptor.

Photosynthetic CO_2 Fixation Measurements on Attached Leaves—Net CO_2 exchange measurements were performed on attached leaves using a portable gas exchange system (LI-6400, Li-Cor Inc, Lincoln, NE) and a homemade red (663 nm) LEDs source. Leaf temperature was maintained at 25 °C, and leaf vapor pressure deficit was maintained around 0.8 kPa. Various O_2 and N_2 concentrations were provided by mixing pure gases. O_2 concentration was measured using a paramagnetic O_2 analyzer (MAIHAK, Hamburg, Germany). This mixing system was also used for fluorescence measurements in attached leaves. Quantum yield of CO_2 fixation in air and under non-photorespiratory conditions (O_2 1.5% (v/v); CO_2 750 μl·liter^{-1}) were calculated from the slope of the linear portion of the light response curve (5 measurements at irradiances between 40 and 80 μmol photons·m^{-2}·s^{-1} and 10 measurements at irradiances between 10 and 100 μmol photons·m^{-2}·s^{-1} for air and non-photorespiratory conditions, respectively).

Electrophoresis and Western Analysis on Chloroplast Fractions—Intact chloroplasts were isolated and purified from leaves using discontinuous Percoll (Amersham Biosciences) gradients as described previously (29). Chloroplasts were osmotically lysed in a solution containing 20 mM MES, pH 6.0, 15 mM NaCl, and 5 mM $MgCl_2$ and centrifuged for 20 min at 35,000 × *g*. Stroma lamellae and grana membranes were separated following a stacking step carried out as described previously (4).

To prepare total insoluble proteins, tobacco leaves (1 g fresh weight) were frozen in liquid nitrogen and ground to a fine powder with a chilled pestle and mortar. The powder was resuspended in a 5-ml extraction buffer (50 mM Tris-HCl, pH 8.0) containing 50 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. After 30 min of stirring (4 °C) and centrifugation (40,000 × *g* for 20 min), the pellet was resuspended in the same buffer containing 1% SDS. After 30 min of stirring (4 °C) and centrifugation (40,000 × *g* for 20 min), proteins contained in the supernatant were precipitated with acetone (80% final concentration).

Denaturing SDS-PAGE was performed as described by Laemmli (30) using 13% acrylamide (w/v) gels. Proteins were transferred onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell) and were probed with the purified anti-At-PTOX serum (24). Immunocomplexes were detected using the chemiluminescence Western blotting kit (Amersham Biosciences).

Transgene Transcript Analysis—RT-PCR analysis of *At-PTOX* transcripts was carried out as described previously (25) by amplifying 363

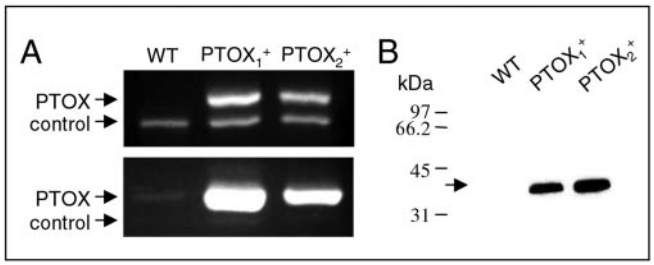

FIG. 1. **At-PTOX transcripts and protein levels in transgenic tobacco lines (PTOX$^+$).** *A*, mRNA levels were determined by RT-PCR amplification of total cellular RNA (*upper panel*) and subsequently re-amplified by a second PCR (*lower panel*). PCR products were separated by electrophoresis on a 1.5% (v/v) agarose gel and visualized by ethidium bromide staining. Total mRNA amounts were standardized beforehand based on fluorescence intensities observed in an ethidium bromide containing RNA gel. Amplification of globin mRNA (added to the RT reaction mix) was used as a control for the RT-PCR to ensure that each sample was reverse-transcribed and amplified equally. *B*, Western analysis was performed on total insoluble leaf proteins (20 μg of proteins) from WT and two PTOX$^+$ transgenic lines, using an anti-At-PTOX antibody.

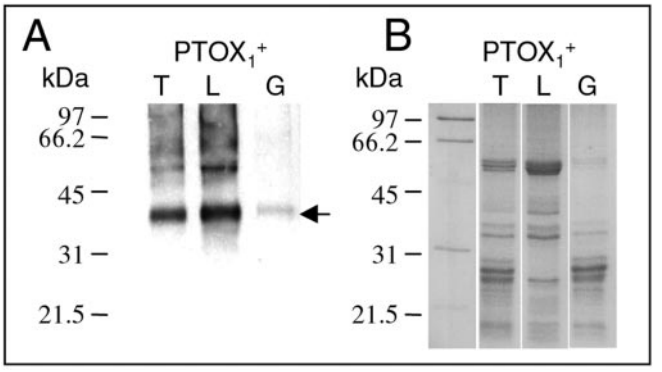

FIG. 2. **Membrane localization of At-PTOX in PTOX$^+$ tobacco chloroplasts.** *A*, Western analysis using an anti-At-PTOX antibody, performed on different membrane fractions prepared from tobacco chloroplasts (5 μg of proteins). *B*, separation of protein fractions (5 μg of proteins) derived from purified chloroplasts by fully denaturing PAGE revealed by Coomassie Brilliant Blue staining. *T*, thylakoid membrane proteins; *L*, stroma lamellae proteins; *G*, grana proteins.

nucleotides of the *At-PTOX* RNAs using the following primers: 5′-GTG CAY TTT GCI GAR AGC TGG AAT G-3′ and 5′-TCA TYG TIT TIC AAT GIT CTG CIT CRT CAT CTC-3′, where Y = C + T, R = A + G, and I = deoxyinosine. The amplification consisted of 30 cycles of 30 s at 94 °C, 20 s at 45 °C, and 20 s at 72 °C. The control was the rabbit globin mRNA (supplied by Invitrogen).

Protein and Chlorophyll Determination—Protein content was determined using a modified Lowry method (Sigma). Chlorophyll content was measured according to the method of Lichtenthaler and Wellburn (31).

RESULTS

Expression of At-PTOX in Tobacco—Transgenic tobacco plants expressing the *At-PTOX* cDNA sequence under the control of the doubled constitutive ^{35}S promoter of the cauliflower mosaic virus were generated by *Agrobacterium*-mediated transformation. Two lines, PTOX$_1^+$ and PTOX$_2^+$, showing a particularly strong expression of the transgene were selected among six transformant lines and were further studied (Fig. 1*A*). Note that although no signal was observed in WT tobacco (Fig. 1*A*, *upper panel*), a faint band was detected after re-amplification (Fig. 1*A*, *lower panel*). We checked that amplified RT-PCR fragments, including the faint band amplified in WT tobacco (Fig. 1*A*), cross-hybridized with the At-PTOX probe by Southern analysis (data not shown). Antibodies raised against At-PTOX were used to characterize At-PTOX expression in tobacco transgenic lines using Western analysis. Both transformant lines showed large amounts of a 41-kDa band corresponding to At-PTOX in total insoluble leaf proteins (25), whereas no signal was observed in wild type (Fig. 1*B*). In both lines, At-PTOX was targeted to the chloroplasts, thanks to the presence of an N-terminal transit peptide (22), and was found to be associated with thylakoid membranes, essentially stroma lamellae, with only small amounts being found in grana (Fig. 2). Subsequent experiments were performed on both PTOX$_1^+$ and PTOX$_2^+$ lines and yielded similar results.

Because PTOX has been reported previously (21, 22) to be involved in carotenoid biosynthesis, the pigment content of transgenic plants was analyzed. High pressure liquid chromatography measurements did not reveal any significant difference in chlorophyll or carotenoid content in WT and PTOX$^+$ extracts (data not shown). In addition, after transfer to high light conditions, similar amounts of xanthophyll cycle carotenoids (violaxanthin, zeaxanthin, and antheraxanthin) were found in both plants. PTOX$_1^+$ and PTOX$_2^+$ plants did not show any particular phenotype, and growth was comparable with WT plants when cultivated under normal conditions (not shown).

Expression of At-PTOX Suppresses the Post-illumination F_0 Fluorescence Increase—When intact WT leaves were illuminated for a few minutes and then placed in the dark, a transient increase in the F_0 chlorophyll fluorescence level occurred (Fig. 3*A*) (see Refs. 32 and 33). The post-illumination fluorescence transient was absent in PTOX$^+$ leaves, and the F_0 fluorescence level rapidly decreased after switching off the light (Fig. 3*B*). As reported previously, the fluorescence increase was absent in Ndh-less mutants (Fig. 3*C*), but interestingly the fluorescence signal decreased more slowly that in PTOX$^+$. The absence of a post-illumination chlorophyll fluorescence increase in Ndh-less mutants was interpreted as the involvement of the Ndh complex in the re-reduction of the PQ pool occurring in the dark after a period of illumination (8, 9, 34). This experiment suggests that like the Ndh complex At-PTOX was able to modulate the redox state of PQ in the dark, most likely by oxidizing reduced plastoquinones. In agreement with this interpretation, when leaf discs were treated with propyl gallate, a potent inhibitor of PTOX (24), a reversal of the loss of the F_0 fluorescence rise was observed (data not shown). Subsequent experiments were designed to characterize the role of At-PTOX in PQ oxidation.

Involvement of At-PTOX in the Dark Oxidation of the PQ Pool—In the experiment described in Fig. 4, chlorophyll fluorescence changes were measured in dark-adapted leaves in response to a saturating light pulse. During a pulse, PS II primary electron acceptors were fully reduced, and chlorophyll fluorescence rapidly reached a maximum level (F_m). After the light pulse, the chlorophyll fluorescence level decreased in the dark, and this decay was related to the reoxidation of PS II primary acceptors (Q_A) in redox equilibrium with the PQ pool. The fluorescence decay was clearly biphasic. The fast phase was similar in WT and PTOX$^+$. On the other hand, the slowly decreasing phase was much faster in PTOX$^+$ than in WT, indicating that PQs were more efficiently reoxidized in transgenic plants. Addition of propyl gallate severely slowed down the fluorescence decay, which came close to that observed in WT leaves (Fig. 4*B*). On the other hand, cyanide (KCN 1 mM) had no significant effect on the fluorescence decay measured in PTOX$^+$ (data not shown). In order to check that PS II acceptors were more reduced in WT than in PTOX$^+$ during the fluorescence decay shown on Fig. 4*A*, a control experiment was performed by flashing a second light pulse 4 s after the first pulse (Fig. 5). Under such conditions, because no non-photochemical

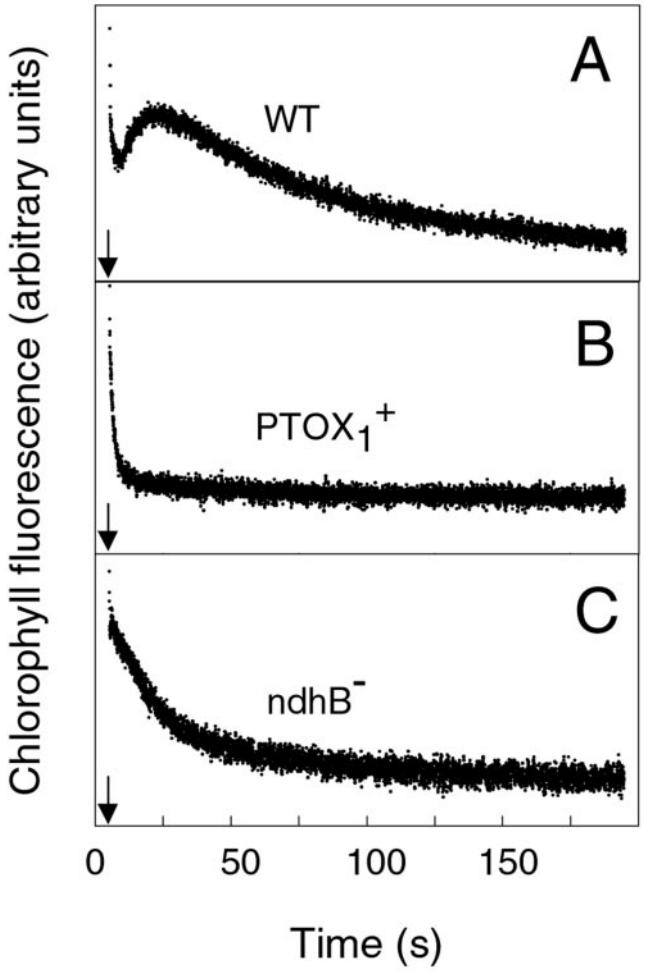

FIG. 3. **Apparent "F_0 rise" of chorophyll fluorescence measured following a light to dark transition in tobacco leaf discs.** Light was switched off when indicated (↓) after a 10-min period of actinic illumination (100 μmol photons. $m^{-2}{\cdot}s^{-1}$). *A*, WT; *B*, $PTOX_1^+$, transgenic tobacco expressing At-PTOX; *C*, *ndhB*$^-$, tobacco plastid transformant inactivated in the *ndhB* gene, and lacking the Ndh complex.

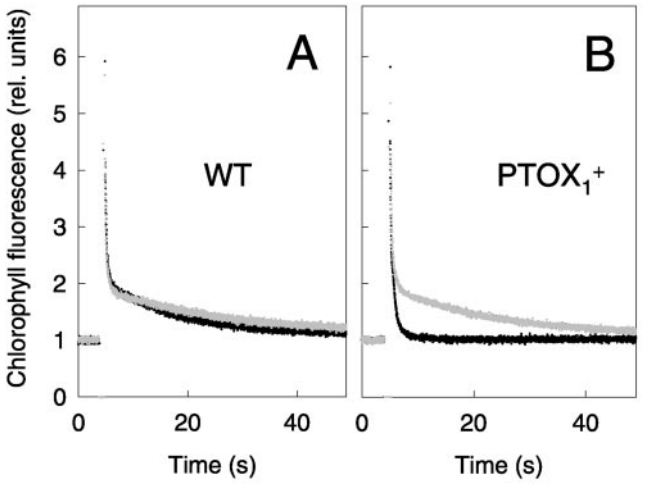

FIG. 4. **Chlorophyll fluorescence decay following a saturating pulse of white light measured on dark-adapted tobacco leaf discs.** Leaf discs, either treated (*gray tracing*) or untreated (*black tracing*) with 1 mM propyl gallate were placed in the dark for 1 h. *A*, WT; *B*, $PTOX_1^+$. Experimental chlorophyll fluorescence values have been normalized to the F_0 value. The maximal photochemical yield of PS II was 0.823 ± 0.013 in WT and 0.820 ± 0.012 in $PTOX^+$ (20 experiments).

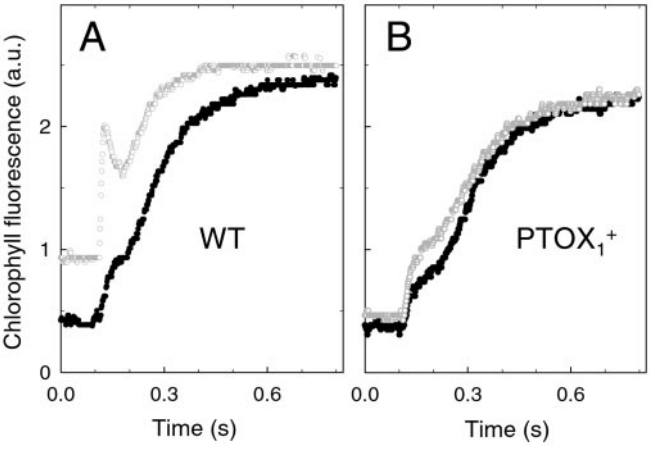

FIG. 5. **Chlorophyll fluorescence measurements in response to a high light intensity (350 μmol photons·$m^{-2}{\cdot}s^{-1}$) in tobacco leaf discs either dark-adapted (*black tracing*) or 4 s following a saturating white light pulse (*gray tracing*).** *A*, WT; *B*, $PTOX_1^+$.

quenching of F_m occurred, the upper area delimited by the fluorescence induction curve reflected the relative pool size of electron acceptors of PS II, mainly the PQ pool (2, 35). Fig. 5*A* shows that in WT leaves, 4 s after the first pulse, PS II acceptors are more reduced than in dark-adapted leaves. In contrast, the redox state of PS II acceptors measured in $PTOX^+$ leaves 4 s after a pulse illumination was close to that measured in dark-adapted leaves (Fig. 5*B*). We concluded from these experiments that At-PTOX was functional in transgenic tobacco leaves and was able to oxidize efficiently reduced PQs following their reduction by a saturating light pulse. We found that propyl gallate slightly (but in a reproducible manner) affected the slow phase of the fluorescence decay measured in WT leaves (Fig. 4*A*), possibly indicating the contribution of a putative tobacco PTOX in PQ oxidation.

At-PTOX Is Active in Thylakoids and Used Molecular O_2 as a Substrate—The activity of PTOX on PQ oxidation was then investigated in chloroplast preparations. Addition of exogenous NADH to osmotically lysed chloroplasts isolated from WT leaves increased the apparent F_0 chlorophyll fluorescence level measured under low non-actinic light, indicating an increase in the redox state of the PQ pool (Fig. 6*A*). Note that in chloroplast preparations, NADH-induced PQ reduction was not mediated by the Ndh complex (Ndh-1), which likely inactivated during the extraction procedure, but rather by an alternative (Ndh-2 like) activity (15, 16, 34). Under aerobic conditions, the NADH-induced fluorescence increase was significantly slower in $PTOX^+$ than in WT chloroplasts (Fig. 6*B*). Addition of propyl gallate increased the chlorophyll fluorescence level in $PTOX^+$ chloroplasts, whereas no significant effect could be detected in WT chloroplasts. Removing O_2 from the sample strongly increased the chlorophyll fluorescence signal in a similar manner in both WT and $PTOX^+$ chloroplasts, and the F_m level corresponded to a full reduction of PQs being rapidly reached (Fig. 6, *A* and *B*). This experiment showed that At-PTOX was functional in isolated tobacco chloroplasts and that the redox state of the PQ pool resulted from a competition between reduction by NADH and oxidation by PTOX.

Mass spectrometric measurements of O_2 exchange were then performed on chloroplast preparations using $^{18}O_2$, to determine which electron acceptor was used during PTOX-mediated PQ oxidation (Fig. 7). In the absence of either cytochrome b_6/f or PS I, an electron flow from PS II to O_2 involving PTOX occurred in *Chlamydomonas* cells (36). When tobacco chloroplasts were treated with DBMIB (a potent inhibitor of the cytochrome b_6/f

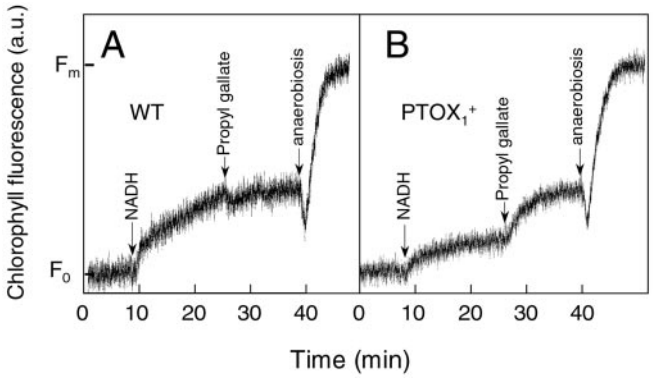

FIG. 6. **Effect of exogenous addition of NADH on the chlorophyll fluorescence level measured in non-actinic modulated light on osmotically lysed tobacco chloroplasts prepared from WT or PTOX$_1^+$ leaves.** When indicated (↓), 2 mM NADH or propyl gallate (1 mM) were added. Anaerobic conditions were obtained by addition of glucose, glucose oxidase, and catalase.

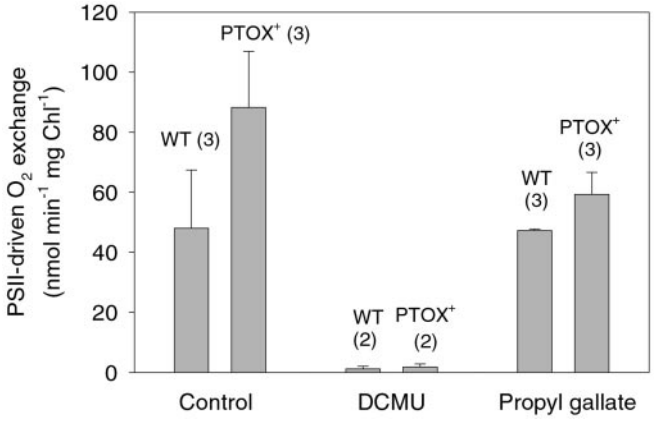

FIG. 7. **Mass spectrometric measurements of light-dependent O_2 exchange in chloroplasts isolated from WT and PTOX$_1^+$ leaves.** O_2 exchange was measured in the presence of $^{18}O_2$ under a light intensity of 150 μmol photons·m^{-2}·s^{-1}. Measurements were performed in the presence of DBMIB (1 μM final concentration). 3-(3,4-Dichlorophenyl)-1,1-dimethylurea or propyl gallate were added at final concentrations of 25 and 8 μM, respectively. Numbers of independent experiments are indicated in *brackets*.

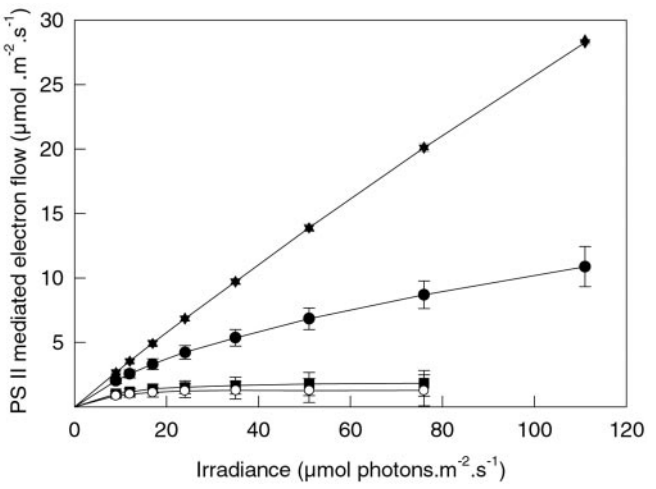

FIG. 8. **Effect of DBMIB on PS II-mediated electron flow measured in stripped leaf discs from WT and PTOX$^+$.** ▲, WT control in water; ▼, PTOX$^+$ control in water; ■, WT treated with 50 μM DBMIB; ●, PTOX$^+$ treated with 50 μM DBMIB; ○, PTOX$^+$ treated with 50 μM DBMIB and 1 mM propyl gallate. Note that because values for WT control and PTOX$^+$ control are very close, corresponding symbols are superimposed. Results obtained for PTOX$_1^+$ and PTOX$_2^+$ were grouped, and *error bars* represent S.D. (six experiments).

complex) and illuminated, simultaneous O_2 production by PS II and O_2 uptake were observed using $^{18}O_2$ and mass spectrometry. PS II activity was higher in PTOX$^+$ than in WT (Fig. 7). Addition of 25 μM of the PS II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea fully suppressed the electron transfer activity, showing the involvement of the PQ pool in both cases. Treatment by propyl gallate largely suppressed the difference in electron transfer activity observed between WT and PTOX$^+$, showing that this difference was likely to be due to the activity of the oxidase. In these experiments, the PS II-mediated electron flow was balanced by a simultaneous increase in the O_2 uptake rate, thus supporting the view that, as in the case of *Chlamydomonas* PTOX, At-PTOX is a true quinol oxidase, using O_2 as an electron acceptor and releasing H_2O as a final product (24).

Measurement of a PS II-mediated Electron Flow to At-PTOX in Leaves—Similar fluorescence experiments were performed in stripped leaf discs treated with DBMIB, measuring chlorophyll fluorescence in order to probe PS II activity (Fig. 8). In the absence of inhibitor, similar electron flow rates were observed at low irradiances both in WT and transgenic leaf discs. In WT leaf discs, DBMIB strongly inhibited linear electron flow (90% inhibition at 75 μmol photons·m^{-2}·s^{-1}), whereas in transgenic leaf discs inhibition by DBMIB was much less pronounced (60% inhibition at 75 μmol photons·m^{-2}·s^{-1}). The DBMIB-insensitive electron flow observed in PTOX$^+$ was inhibited by propyl gallate (Fig. 8) and reached basal rates measured in WT discs treated with propyl gallate. This experiment showed that in leaves placed under low light intensity, when linear electron flow to PS I was inhibited, a significant part of PS II-driven electron flow (about 35% of the maximal electron flow to PS I) can be directed toward PTOX and O_2.

Involvement of PTOX during Photosynthesis—We were then interested to determine whether the activity of At-PTOX, which can be evidenced either in the dark (Figs. 3 and 4) or in the light in the absence of functional electron transfer to PS I (Fig. 8), could be observed in the light during normal conditions of photosynthesis. During a dark to light induction of photosynthesis, typical variations in chlorophyll fluorescence were observed (37). Under low light intensity, these variations reflected changes in the electron transfer rate occurring during the activation of photosynthesis. The transient increase in fluorescence commonly observed in WT during the induction phase reflected the transient accumulation of plastoquinols due to the initial absence of PS I electron acceptors. In fact, an activation of the PS I acceptor side and of Calvin cycle enzymes was generally required to initiate CO_2 assimilation and further reoxidize NADPH. This transient was almost completely abolished in PTOX$^+$ at the lowest irradiance (8 μmol photons·m^{-2}·s^{-1}, Fig. 9*A*), indicating a highly efficient plastoquinol oxidation before the activation of PS I. After a few minutes of illumination, both F_s and $F_{m'}$ values were identical in WT and PTOX$^+$. At this low irradiance, $F_{m'}$ was close to F_m, showing the absence of non-photochemical quenching. At higher light intensity (50 μmol photons·m^{-2}·s^{-1}), a difference between WT and PTOX$^+$ was also observed, F_s values remained lower in PTOX$^+$ than in WT during the first 3 min of illumination (Fig. 9*B*). When illumination was prolonged, the decrease in F_s was more pronounced in WT, and after 10 min reached a lower level than in PTOX$^+$. It should be noted that variations in F_s values were accompanied by concomitant changes in $F_{m'}$ (Fig. 9*B*). As a consequence, both non-photochemical (qN) and photochemical (qP) quenching parameters were lower in PTOX$^+$ than in WT after 10 min of illumination. This effect on qN and qP was also observed at higher irradi-

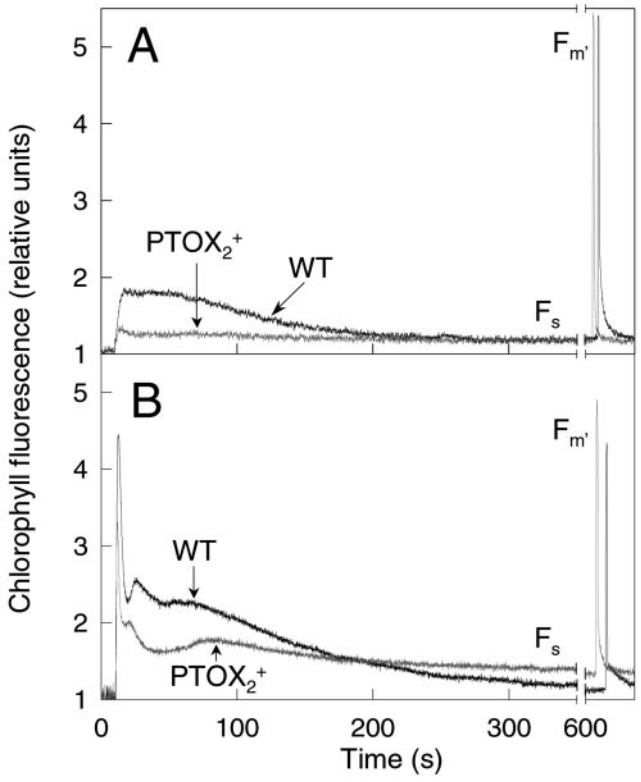

FIG. 9. **Chlorophyll fluorescence induction curves measured during a dark to light transition on attached leaves of WT and $PTOX_2^+$ tobacco plants.** After 10 min a saturating pulse was used to determine $F_{m'}$ and to calculate both qP and qN values. *A*, at an irradiance of 8 μmol photons·m^{-2}·s^{-1}; qP and qN values were identical for WT and $PTOX^+$; *B*, at an irradiance of 50 μmol photons·m^{-2}·s^{-1}. Experimental chlorophyll fluorescence values have been normalized to the F_0 value. qP and qN values (0.956 and 0.212 in WT, respectively) were significantly decreased in $PTOX^+$ (0.914 and 0.111, respectively).

ances (for example at 750 μmol photons·m^{-2}·s^{-1}; Table I). However, at all irradiances, after 1 h of illumination when a steady state was reached, qN and qP of WT and $PTOX^+$ became identical (Table I). Despite these fluctuations in fluorescence quenchings, no significant differences in PS II photochemical yields, measured either at 10 or 60 min, could be evidenced between WT and $PTOX^+$ (Table I). Moreover, measurements of net CO_2 gas exchange at steady state showed no significant difference in quantum yield of CO_2 fixation in air as well as under non-photorespiratory conditions (Table II). In WT and $PTOX^+$, rates of CO_2 fixation measured at saturating irradiance were also similar (Table II).

TABLE I

PS II photochemical yield, chlorophyll fluorescence quenching parameters (qN and qP) measured in WT and $PTOX^+$ attached leaves

Measurements were performed 10 and 60 min after the onset of illumination (750 μmol photons · m^{-2} · s^{-1}). Values are means ± S.D. of 3 experiments.

Chlorophyll fluorescence parameters		WT	$PTOX^+$
$(F_{m'} - F_s)/(F_{m'}$		0.333 ± 0.023	0.339 ± 0.016
After 10 min	qP	0.731 ± 0.011	0.632 ± 0.022
illumination	qN	0.670 ± 0.018	0.593 ± 0.022
$(F_{m'} - F_s)/F_{m'}$		0.350 ± 0.026	0.324 ± 0.032
After 60 min	qP	0.743 ± 0.018	0.759 ± 0.009
illumination	qN	0.599 ± 0.025	0.622 ± 0.033

TABLE II

Quantum yield of CO_2 fixation (Φ_{CO2}) and maximal rate of CO_2 assimilation measured in attached leaves of WT and PTOX tobacco

The maximal rate was measured under a saturating irradiance of 750 μmol photons · m^{-2} · s^{-1}. Quantum yields were measured under photorespiratory conditions (air) and under non-photorespiratory conditions (O_2 1.5% (v/v); CO_2 750 μl · $liter^{-1}$). Number of experiments is indicated in parentheses.

	WT	$PTOX^+$
Φ_{CO2} (air)	0.0481 ± 0.0038 (3)	0.0440 ± 0.0056 (3)
Φ_{CO2} (1.5% O_2; 750 μl · $liter^{-1}$ CO_2)	0.0783 ± 0.0023 (3)	0.0801 ± 0.0021 (3)
Maximal CO_2 assimilation (μmol · m^{-2} · s^{-1})	13.7 ± 0.7 (5)	13.6 ± 1 (5)

DISCUSSION

We have shown in this paper that when expressed in tobacco, At-PTOX is targeted to the chloroplasts and functions as a PQ oxidase. The activity of At-PTOX could be evidenced in intact leaves, following either photochemical or non-photochemical reduction of PQs and also in thylakoids, when PQs were reduced by exogenous NADH. Based on chlorophyll fluorescence and mass spectrometric measurements performed on thylakoids, we propose that At-PTOX drives PQ oxidation using molecular O_2 as a terminal electron acceptor. This agrees with previous conclusions reached from mass spectrometric measurements on *Chlamydomonas* mutants deficient in PS I (24). Because the Ndh complex is involved in the non-photochemical reduction of the PQ pool (8, 9, 34) (see Fig. 3*C*) and At-PTOX is involved in its non-photochemical oxidation, we conclude that a chlororespiratory electron transfer involving the plastid Ndh complex, the PQ pool, and At-PTOX occurs from NAD(P)H to O_2 in chloroplasts of transgenic tobacco expressing At-PTOX. In the dark, the redox status of PQs therefore depends on an equilibrium between its reduction by the Ndh complex and oxidation by PTOX.

In thylakoid membranes, PS I reaction centers and ATPase complexes are essentially located in stroma lamellae, whereas PS II are restricted to grana, cytochrome b_6/f complexes being found in both types of membranes. Like the Ndh complex (11, 38, 39), At-PTOX was found mainly in stroma lamellae, indicating that chlororespiration is restricted to stroma lamellae and is absent in granal thylakoids. Previously, the involvement of a propyl gallate-sensitive PQ oxidase in chlororespiration had been evidenced in *Chlamydomonas* cells (24). It was proposed that an At-PTOX homologue was functional in *Chlamydomonas* thylakoid membranes (24), but the corresponding gene has not yet been identified (16). In higher plants, first evidence for the existence of chlororespiration was based on the effect of respiratory inhibitors such as cyanide (40) or CO (41). Such effects cannot be explained by the inhibition of PTOX, because this protein was reported to be insensitive to these compounds (24, 25). This was confirmed in this study by the insensitivity to cyanide of the slow phase of the chlorophyll fluorescence decay. Therefore, the effects of respiratory inhibitors such as cyanide or CO more likely result from the inhibition of mitochondrial respiration that has been reported to affect the redox state of the PQ pool due to the existence of redox interactions between chloroplasts and mitochondria (16, 42). Such effects may alternatively reflect the existence of an alternative PQ oxidation pathway sensitive to cyanide and CO. In this respect, Fig. 7 indicates the existence in chloroplasts of a propyl gallate-insensitive mechanism for PQ oxidation. Recently, Casano *et al.* (6), studying a reconstituted system containing the Ndh complex and a plastidial hydroquinone peroxidase, proposed the existence of a PQ oxidation pathway using hydrogen peroxide as a terminal acceptor.

In addition to an involvement in dark reactions, we have

shown that At-PTOX may interact with photosynthetic electron transport reactions in illuminated leaves. In WT plants, a transient over-reduction of photosynthetic electron carriers occurs during the induction phase of photosynthesis. This is due to the fact that the photosynthetic carbon reduction cycle is not operative in the dark, because some of the enzymes of the cycle require light-induced activation by reduced thioredoxins (43). In transgenic tobacco plants expressing At-PTOX, the transient over-reduction of photosynthetic electron carriers is greatly decreased, indicating that electrons are diverted to O_2 via PTOX. This suggests that PTOX can potentially prevent over-reduction of PQs in the light. In plant mitochondria, alternative oxidase has been suggested to function as an "energy overflow," its activity being increased when the cytochrome pathway is saturated with electrons (44). Overexpression of alternative oxidase in this organelle has been shown to limit the generation of reactive oxygen species by preventing over-reduction of electron carriers (45). It should be noted, however, that expression of At-PTOX did not result in increased resistance of transgenic lines to photoinhibition (data not shown).

Differences in qN and qP values between WT and $PTOX^+$ were transitorily observed during the 10–30-min period of illumination, whereas the photochemical yield of PS II remained identical in both WT and $PTOX^+$. The fact that both photochemical yield of PS II and rate of CO_2 fixation are identical suggests that at the end of the transitory induction period of photosynthesis, the oxidase function of PTOX does not contribute to drive significant electron flow compared with photosynthetic carbon reduction and oxidation cycles. On the other hand, lower qN and qP in $PTOX^+$ between the initial induction period and steady state suggests that the pH gradient is lower and that PS II acceptors (Q_A) are more reduced compared with the WT. A lower pH gradient could indicate that cyclic electron reactions around PS I are down-regulated in $PTOX^+$. Cyclic electron reactions around PS I have been reported to be controlled by the redox poise of some electron carriers; this effect was possibly mediated by molecular O_2 (46). Overexpression of PTOX, by modifying the redox poise of intersystem electron carriers, may perturb the establishment of cyclic electron transfer reactions. Interestingly enough, a role of chlororespiration in the control of cyclic electron flow around PS I was recently deduced from photoacoustic measurements performed in leaves under low O_2 concentration (12). The fact that chlororespiration and cyclic electron transfer reactions around PS I operate within the same membranes (stroma lamellae, see Ref. 16) further strengthens the hypothesis of a functional link between these two activities.

At steady state, qP and qN values were similar in WT and $PTOX^+$, indicating that both the redox state of Q_A and the pH gradient reached similar levels. This may indicate that at steady state the contribution of cyclic electron flow around PS I is decreased compared with its high activity during the induction phase when terminal electron acceptors are not fully available. Alternatively, this effect might reflect the involvement of regulatory mechanisms that could be turned on under these conditions. For instance, the expression of some nuclear genes, like *cab* genes encoding light harvesting complex apoprotein, has been shown to be controlled by the redox state of PQs (47). The higher reduction of the PQ pool observed in $PTOX^+$ during the induction of photosynthesis may trigger such long term adaptation processes and explain why similar pH gradients and Q_A redox state are finally reached at steady state in both types of plants. Analysis of gene expression in $PTOX^+$ plants should inform us of the possible existence of such adaptive mechanisms.

If the role of At-PTOX in PQ oxidation could be demonstrated in transgenic tobacco, the involvement of a functional PTOX in WT tobacco appears more difficult to establish. A faint band, specific to the native *ptox* transcripts, was amplified in WT tobacco by RT-PCR (Fig. 1*A*). However, by using an antibody raised against At-PTOX, no signal corresponding to native PTOX was detected in insoluble proteins prepared from WT tobacco leaves (Fig. 1). This may be due to the fact that either the antibody raised against the *Arabidopsis* enzyme does not cross-react with the tobacco enzyme or that the native enzyme is present in too small an amount to be detected. The latter hypothesis is the most probable, because this antibody cross-reacts with chromoplast preparations from pepper, another Solanacae species (25), and also with chloroplast preparations from *C. reinhardtii* (24). In this respect, a doublet that may correspond to the native tobacco PTOX was detected in purified stroma lamellae preparations probed with the *Arabidopsis* antibody (data not shown). It should be noticed that the plastid Ndh complex, the other probable component of chlororespiration, has been reported to be present in leaves in very low amounts (4, 39). The slight effect of propyl gallate on the slow phase of the chlorophyll fluorescence decay measured in WT leaves (Fig. 3), may reflect a contribution of the native tobacco PTOX to the oxidation of PQs. In agreement with this interpretation, it has been reported recently (12) that in tobacco leaves the re-reduction rate of the oxidized primary electron donor in PS I (P_{700}^+) is increased by propyl gallate. This effect was interpreted as the re-routing of electrons toward PS I when the putative tobacco plastid terminal oxidase is inhibited. Since PTOX most likely represents a minor component of thylakoid membranes, at least when plants are grown under normal conditions, a regulatory role (for instance in the control of cyclic electron flow) seems more probable than a direct bioenergetic role. However, more work remains to be done to determine clearly the involvement of native PTOX in leaves.

Different lines of evidence suggest that PTOX might become more abundant at particular developmental stages or under particular growth (or stress) conditions. In higher plant chloroplasts, the role of PTOX in carotenoid biosynthesis has been demonstrated from the analysis of *Arabidopsis* and tomato mutants (21, 22, 25). The variegated phenotype of the *Arabidopsis* mutant *immutans* was explained by an involvement of PTOX in phytoene desaturation, an important step in carotenoid biosynthesis occurring during the early stage of the greening process (21, 22). As suggested previously (16, 19), native PTOX might be more abundant in non-green plastid under conditions where the photosynthetic apparatus is not functional. High amounts of PTOX were reported in achlorophyllous membranes prepared from chromoplasts of red pepper fruits, where carotenoid biosynthesis is particularly active (25). Overexpression of At-PTOX did not influence the leaf carotenoid content, thus indicating that the PTOX level is not a limiting factor regulating carotenoid biosynthesis. Interestingly, the *IM* (or *PTOX*) promoter was shown to be active, and *IM* mRNAs were expressed ubiquitously in *Arabidopsis* tissues and organs throughout development, arguing in favor of a more global role for this protein in plastid metabolism (48).

In C_4 plants, subunits of the Ndh complex have been reported to be much more strongly expressed in bundle sheath chloroplasts than in mesophyll chloroplasts (49). In bundle sheath chloroplasts, only low levels of PS II are detected. In these cells, ATP required for CO_2 fixation is generated by cyclic electron transport around PS I. Interestingly, it was recently reported that bundle sheath cells from C_3 leaves have photosynthesis features close to those of C_4 leaves (50). It will be interesting to determine whether *ndh* and *ptox* genes are more strongly expressed in bundle sheath chloroplasts of C_3 plants

than in mesophyll cells and participate in the regulation of cyclic electron transfer reactions around PS I.

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Conclusion Générale

Dans le but de comprendre le rôle du complexe plastidial Ndh, nous avons obtenu des plants de tabac transplastomiques dont le gène *ndhB*, codant pour une des sous-unités, a été inactivé. L'étude de transformants homoplasmiques nous a permis de montrer que le complexe Ndh, normalement présent dans les thylacoïdes lamellaires des plantes de type sauvage, est absent chez les plantes transplastomiques. Par des mesures de fluorescence de chlorophylle nous avons montré que le complexe Ndh est fonctionnel chez les plantes de type sauvage et qu'il est impliqué dans la réduction non photochimique du pool de PQ. Les plantes transplastomiques dépourvues du complexe Ndh présentent un développement et une croissance similaires aux plantes de type sauvage dans la plupart des conditions environnementales étudiées. Par contre, dans des conditions de déficit hydrique modéré, les plantes mutantes présentent une capacité d'assimilation photosynthétique et une croissance réduites, ces effets étant supprimés en présence d'une concentration elevée en CO_2. En traitant des plants de tabac par de l'acide abscissique, une hormone provoquant la fermeture stomatique, les mêmes retards de croissance ont été observés chez les mutants. Nous avons conclu de ces expériences que les différences observées étaient liées à une teneur interne en CO_2 faible, conditions favorisant la photorespiration. Nous avons fait l'hypothèse que la demande en ATP, accrue en conditions de photorespiration élevée, n'était pas satisfaite chez les plantes mutantes et que le complexe Ndh était impliqué dans la fourniture d'ATP, probablement à travers sa participation au transfert cyclique des électrons autour du PS I.

Pour tester l'hypothèse d'une participation du complexe Ndh au transfert cyclique des électrons autour du PS I, nous avons étudié l'effet de l'antimycine A, composé connu pour inhiber *in vitro* les photophosphorylations cycliques. Nous avons observé que l'activité photosynthétique de disques foliaires de plantes mutantes était significativement plus sensible à l'action inhibitrice de l'antimycine A que celle de plantes de type sauvage. Nous avons proposé l'existence de deux voies de transfert cyclique autour du PS I, l'une insensible à l'antimycine A et impliquant le complexe Ndh et l'autre, sensible à l'antimycine A, impliquant probablement la FQR. En se basant sur l'action inhibitrice de l'antimycine A mesurée dans des conditions où la composition atmosphérique est variable, nous avons conclu que dans des conditions où la demande en ATP est faible (peu de photorespiration), chacune des voies peut indépendamment satisfaire la demande. Par contre, dans des conditions où la

demande en ATP est plus forte (forte photorespiration), le fonctionnement des deux voies est nécessaire pour assurer une croissance optimale.

Afin de mettre en évidence de manière plus directe le transfert cyclique des électrons autour du PS I, nous avons eu recours à la spectrométrie photoacoustique. Dans un premier temps, en accord avec les données publiées antérieurement (Herbert et al., 1990 ; Havaux, 1991; Malkin et al., 1992), seules de faibles activités de stockage photochimique attribuables au transfert cyclique des électrons autour du PS I ont pu être mises en évidence. En se basant sur l'hypothèse que l'activité du transfert cyclique pourrait être contrôlée par l'état rédox de certains transporteurs d'électrons, nous avons effectué des mesures de stockage d'énergie photochimique dans des conditions où le pool de PQ est partiellement réduit. Dans ces conditions, l'activité cyclique est fortement stimulée. L'étude des plantes mutantes nous a permis de distinguer deux voies de transfert cyclique, dont une voie rapide, impliquant le complexe Ndh.

Dans le but de déterminer si PTOX, une oxydase impliquée dans la biosynthèse des caroténoïdes récemment découverte dans les chloroplastes, pourrait jouer un rôle dans les réactions de transfert d'électrons au sein des chloroplastes nous avons produit des plantes de tabac transgéniques surexprimant la protéine d'*Arabidopsis thaliana.* L'étude des plantes transgéniques produites nous a permis de montrer que PTOX est impliquée dans l'oxydation non-photochimique du pool de PQ et utilise l'oxygène moléculaire comme accepteur terminal d'électrons. Nous avons montré qu'à l'obscurité, le niveau rédox du pool de PQ résulte d'un équilibre entre une voie de réduction non photochimique impliquant le complexe Ndh et une voie d'oxydation non photochimique impliquant PTOX. Nous avons conclu de l'ensemble de ces expériences à l'existence, dans les chloroplastes des végétaux supérieurs, d'une chaîne chlororespiratoire de transporteurs d'électrons. Bien que les transferts d'électrons de type chlororespiratoires soient limités, PTOX fonctionne à la lumière comme une "soupape de sécurité" permettant de limiter la réduction transitoire du pool de PQ. La surexpression de PTOX perturbe l'établissement de quenchings de fluorescence de la chlorophylle reliés à l'établissement du gradient de protons. Nous avons émis l'hypothèse que l'activité du transfert cyclique des électrons autour du PSI était affectée chez ces mutants. Plusieurs hypothèses ont été avancées pour expliquer les phénomènes de régulations impliqués dans l'établissement du transfert cyclique. Le PS II, en réduisant les transporteurs d'électrons situés entre les deux photosystèmes pourrait enclencher le transfert cyclique (Heber et Walker, 1992). Il a également été proposé que l'oxygène moléculaire participe à ces mécanismes, le transfert cyclique des électrons autour du PS I mesuré sur des chloroplastes étant stimulé en

conditions de faibles concentrations en oxygène moléculaire (Ziem-Hanck et al., 1980 ; Hormann et al., 1994). Ces phénomènes ont été initialement interprétés par une possible compétition entre transfert cyclique et pseudocyclique (Hormann et al., 1994). D'après l'ensemble de nos expériences, nous proposons que l'activité de la chlororespiration, en permettant un contrôle fin de l'état rédox de certains transporteurs d'électrons, régule l'activité du transfert cyclique autour du PS I (Figure 1). Cette hypothèse est compatible avec la localisation des complexes photosynthétiques au sein des membrane thylacoïdiennes, le complexe Ndh et PTOX étant localisés comme les PS I, les ATPases et une partie des complexes cyt b_6/f dans les lamelles du stroma. Au cours du transfert linéaire d'électrons le couplage entre le PSII et le PSI s'opère grâce à la diffusion latérale des plastocyanines des thylacoïdes granaires vers les thylacoïdes lamellaires. La diffusion des plastocyanines étant beaucoup plus rapide que celle des plastoquinones, ceci aboutit à l'existence *de facto* de deux pools de PQ relativement indépendants. L'état rédox du pool de PQ des lamelles du stroma est donc directement tributaire de l'activité chlororespiratoire. Un déséquilibre transitoire entre les phénomènes de réduction et d'oxydation non photochimique du pool de PQ peut mener à une réduction des transporteurs des lamelles et ainsi stimuler l'activité de transfert cyclique des électrons autour du PS I. Mais l'activité du transfert cyclique des électrons peut être inhibée lorsque la chaîne de transfert d'électrons entre les deux photosystèmes est totalement réduite (Ziem-Hanck et al., 1980). PTOX pourrait jouer le rôle de "soupape de sécurité" évitant ainsi l'engorgement de la chaîne de transfert d'électrons et l'inhibition du transfert cyclique. Ainsi, les flux d'électrons du pool de PQ vers PTOX, mêmes s'ils sont relativement faibles, pourraient réguler des transfert d'électrons quantitativement plus importants du transfert cyclique.

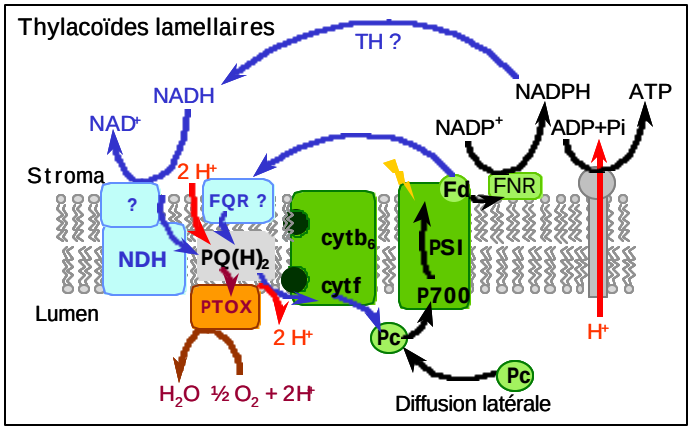

Figure 1. Modèle proposé présentant les interactions entre transfert cyclique des électrons autour du PS I et chlororespiration au sein des thylacoïdes lamellaires des plantes supérieures. Le transfert linéaire des électrons est présenté en noir, le transfert cyclique en bleu, la chlororespiration en marron et les translocations de protons en rouge. La nature moléculaire de la FQR reste inconnue. La présence d'une activité transhydrogénase (TH) reste à établir. L'orientation de PTOX vers le lumen est arbitraire.

A l'issue de ce travail de thèse, diverses questions restent posées. L'existence d'un flux d'électrons de type chlororespiratoire transitant à travers le complexe Ndh, le pool de PQ et l'activité quinol oxydase de PTOX est maintenant démontré. Cependant, le rôle physiologique de cette activité reste à établir. A ce titre, l'étude des plantes transgéniques surexprimant PTOX mériterait d'être approfondie afin de déterminer si le transfert cyclique des électrons est réellement affecté chez ces plantes. L'étude des capacités photosynthétiques sous des conditions où l'activité photorespiratoire varie permettrait d'étudier la réponse des plantes transgéniques à des variations dans la demande en ATP.

Il a été démontré que l'état rédox de la chaîne de transport d'électrons intersystème et plus particulièrement du pool de PQ et du cyt b_6/f permet un contrôle de l'expression de certains gènes photosynthétiques. Les phénomènes impliqués pourraient notamment jouer un rôle dans les processus d'adaptation de l'appareil photosynthétique lors de changements des conditions environnementales (Allen , 1992). La chlororespiration, de par son activité de réduction et d'oxydation du pool de PQ, pourrait être indirectement impliquée dans le contrôle de l'expression de certains gènes photosynthétiques. La régulation différentielle de l'expression de certains gènes pourrait être suivie chez des mutants *ndhB*⁻ ou $PTOX^+$ (et $PTOX^-$) à l'aide de puces à ADN. A ce titre, il pourrait être intéressant d'effectuer des croisements entre les plantes mutantes $ndhB^-$ et $PTOX^+$ qui ont été obtenus sur le même matériel végétal afin d'obtenir des plantes au sein desquelles le niveau redox du pool de PQ tendrait à être très oxydé.

La voie de transfert cyclique des électrons autour du PSI qui est sensible à l'antimycine A implique potentiellement une activité FQR. Cependant, cette enzyme n'est pas caractérisée au niveau moléculaire. D'autre part, des voies alternatives d'oxydation du pool de PQ ont été décrites, impliquant une activité hydroquinone peroxydase récemment détectée au sein des thylacoïdes des végétaux supérieurs (Zapata et al., 1998) et capable de réoxyder le pool de PQ sur des systèmes membranaires reconstitués (Casano et al., 2000). Le clonage des gènes codant pour ces deux enzymes s'avère donc une étape importante de l'étude des processus de réduction et d'oxydation non photochimique du pool de PQ. Dans ce cadre, les mêmes puces à ADN pourrait permettre de détecter des voies alternatives d'oxydation qui seraient réprimées chez le mutant de tabac $PTOX^+$ ou surexprimées chez les mutants Immutans ($PTOX^-$) d'*Arabidopsis thaliana* et des voies alternatives de réduction du pool de PQ qui seraient surexprimées chez les mutants ndhB⁻.

Si nos études ont permis de conclure à l'implication du complexe Ndh dans les réactions de transfert cyclique des électrons autour du PSI, le rendement énergétique de ce mécanisme

demeure inconnu. En effet, l'efficacité du transfert cyclique, c'est-à-dire la quantité d'ATP produite par nombre d'électrons transférés dépend du caractère électrogénique ou non-éléctrogénique du complexe Ndh. L'étude de ces mécanismes de translocation de protons du stroma vers le lumen pourrait s'effectuer *in planta* à l'aide de sondes fluorescentes marquant les variations de pH. Le complexe Ndh et PTOX étant peu abondants, l'étude de l'électrogénicité du complexe Ndh et de la chlororespiration pourrait être réalisée *in vitro* dans des systèmes reconstitués ou dans des systèmes ou le complexe Ndh est abondant et les complexes photosynthétiques absents (au sein des étioplastes, par exemple).

La nature des sous-unités formant le domaine catalytique et la nature du substrat du complexe Ndh restent à déterminer. Il est à noter qu'une démarche de double hybride a été entreprise au cours de cette thèse. En criblant une banque d'ADNc d' *Arabidopsis thaliana* en utilisant des protéines appâts correspondant aux sous-unités solubles du complexe Ndh telles que les peptides NDH-H,I,J et K, nous avions pour objectif de détecter d'éventuelles sous-unités solubles du complexe Ndh codées par le génome nucléaire et impliquées dans l'activité catalytique. Cependant, cette stratégie n'a pas abouti. Les sous-unités solubles du complexe Ndh exprimées en fusion avec la protéine Gal4 sont incapables d'interagir entre elles dans un organisme hétérologue tel que la levure. Une approche *in planta* semble plus prometteuse. Ainsi, un crible de mutants nucléaires pourrait s'effectuer à travers l'analyse de la fluorescence de la chlorophylle sur des jeunes plantules. L'absence de pic transitoire d'augmentation de la fluorescence après une période d'éclairement serait le critère choisi pour obtenir des mutants affectés dans la réduction non photochimique du pool de PQ et permettre de trouver des sous-unités "manquantes" du complexe Ndh.

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